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Mitochondrial depolarization following hydrogen sulfide exposure in erythrocytes from a sulfide-tolerant marine invertebrate

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Summary

Sulfide-tolerant marine invertebrates employ a variety of mechanisms to detoxify sulfide once it has entered their bodies, but their integumentary, respiratory epithelium and circulatory cells may still be exposed to toxic sulfide concentrations. To investigate whether sulfide exposure is toxic to mitochondria of a sulfide-tolerant invertebrate, we used the fluorescent dyes JC-1 and TMRM to determine the effect of sulfide exposure on mitochondrial depolarization in erythrocytes from the annelid *Glycera dibranchiata*. In erythrocytes exposed to 0.11–1.9 mmol l^{-1} sulfide for 1 h, the dyes showed fluorescence changes consistent with sulfide-induced mitochondrial depolarization. At the highest sulfide concentration, the extent of depolarization was equivalent to that caused by the mitochondrial uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Even when induced by as little as 0.3 mmol l^{-1} sulfide, the depolarization was not reversible over a subsequent 5 h recovery period. The mechanism of toxicity was likely not *via* inhibition of cytochrome *c* oxidase (COX), since other COX inhibitors and other mitochondrial electron transport chain inhibitors did not produce similar effects. Furthermore, pharmacological inhibition of the mitochondrial permeability transition pore failed to prevent sulfide-induced depolarization. Finally, increased oxidation of the free radical indicators H_2DCFDA and MitoSOX™ in erythrocytes exposed to sulfide suggests that sulfide oxidation increased oxidative stress and superoxide production, respectively. Together, these results indicate that sulfide exposure causes mitochondrial depolarization in cells of a sulfide-tolerant annelid, and that this effect, which differs from the actions of other COX inhibitors, may be *via* increased free radical damage.

Key words: hydrogen sulfide, *Glycera dibranchiata*, mitochondria, coelomocyte, free radical.

Introduction

Animals in habitats such as mudflats, marshes, cold seeps and hydrothermal vents can be periodically or continuously exposed to sulfide (representing the sum of H_2S, HS^- and S^{2-}), which is a lethal, highly reactive toxin that is well known as a reversible inhibitor of cytochrome *c* oxidase (COX; EC 1.9.3.1), i.e. the terminal enzyme of the mitochondrial electron transport chain (Dorman et al., 2002; Khan et al., 1990; Nicholls and Kim, 1982). Sulfide, cyanide and azide each inhibit COX (Lloyd et al., 1982; Nicholls and Kim, 1982; Smith et al., 1977), although perhaps *via* different specific mechanisms (Nicholls, 1975). Inhibition of COX activity by 50% occurs *in vitro* at 1–5 μmol l^{-1} sulfide concentrations and *in vivo* at 1–40 μmol l^{-1} sulfide concentrations (for a review, see Bagarinao, 1992). Sulfide has additional potential mechanisms of toxicity, however, including inhibition of almost 20 other enzymes (Bagarinao, 1992), formation of sulfhemoglobin (e.g. Bagarinao and Vetter, 1992; Kraus et al., 1996; Völkel and Berenbrink, 2000), production of free radicals (Chen and Morris, 1972; Eghbal et al., 2004; Tapley et al., 1999), interaction with neuronal signalling pathways (Rosenegger et al., 2004), and potentially even as a neuromodulator itself (e.g. Gainey and Greenberg, 2005; Julian et al., 2005; reviewed by Kimura, 2002). Nonetheless, COX inhibition is generally considered to be the lethal mechanism, largely because many of these additional effects only occur at much higher sulfide concentrations, at least *in vitro* (Bagarinao, 1992; Beauchamp et al., 1984; Khan et al., 1990). Sulfide-adapted animals do not appear to have sulfide-insensitive COX (Hand and Somero, 1983; Powell and Arp, 1989), and instead their sulfide-tolerance is thought to result from sulfide detoxification mechanisms (Grieshaber and Völkel, 1998). However, even with detoxification, epithelial tissues and blood cells of these animals may still experience sufficient sulfide exposure to cause cellular toxicity, even if only transiently (Völkel and Grieshaber, 1994; Wohlgemuth et al., 2000).

In normally functioning mitochondria, oxidative phosphorylation maintains an electrochemical proton (H^+) gradient, and therefore a mitochondrial electrical potential ($\Delta\Psi m$), which drives the ATP synthase (F_1F_O-ATP synthase). In mammalian cells, reduced $\Delta\Psi m$ can result from increased

cellular demand for ATP, mitochondrial outer membrane permeabilization (MOMP) and uncouplers of oxidative phosphorylation (Bernardi et al., 2001; Green and Kroemer, 2004; Ly et al., 2003). Although it might be assumed that inhibition of oxidative phosphorylation would directly decrease $\Delta\Psi$m, this is not necessarily true. For example, azide and cyanide *in vitro* decrease $\Delta\Psi$m in some mammalian cells (Feeney et al., 2003; Jensen et al., 2002; Prabhakaran et al., 2002), but other mammalian cells show only a small reduction in $\Delta\Psi$m during cyanide exposure unless inhibitors of glycolysis are added simultaneously (Lawrence et al., 2001). With regard to sulfide exposure, one study has reported that isolated rat hepatocytes exposed to 0.5 mmol l^{-1} sulfide *in vitro* lost over 50% of $\Delta\Psi$m within 1 h, and that this effect was reduced when the cells were supplemented with glycolytic substrate (Eghbal et al., 2004). In contrast, another study on the same cell type reported that supplementation with glycolytic substrate had no effect on cell survival during sulfide exposure, although it did increase cell survival during cyanide exposure (Thompson et al., 2003). Therefore, whether sulfide exposure causes cellular toxicity *via* decreased $\Delta\Psi$m is unclear. Furthermore, short-term sulfide exposure in mice under controlled conditions greatly decreased metabolic rate but had no apparent long-term effect on the animal's health, indicating that inhibition of oxidative phosphorylation by sulfide does not necessarily lead to cell death (Blackstone et al., 2005). Whether $\Delta\Psi$m was affected in the mice was not determined.

One possible mechanism of sulfide-induced mitochondrial depolarization is MOMP, which in mammalian cells can result from exposure to heavy metals, reactive oxygen species (ROS) and a variety of other toxins (Green and Kroemer, 2004). Two separate mechanisms appear to induce MOMP: (1) opening of the mitochondrial permeability transition (PT) pore, which permeabilizes the mitochondrial inner membrane, leading to dissipation of $\Delta\Psi$m, influx of water into the matrix and eventual rupturing of the outer membrane, and (2) formation of a pore that permeabilizes the mitochondrial outer membrane (Green and Kroemer, 2004). In either case, MOMP leads inexorably to cell death through the release of pro-apoptotic factors from the mitochondrial inter-membrane space into the cytosol (He and Lemasters, 2002; Hunter et al., 1976; Kim et al., 2003a; Ly et al., 2003). In rat hepatocytes *in vitro*, the PT pore inhibitors cyclosporine A (CsA) and trifluoperazine (TFP) decreased sulfide-induced cell death by 50% compared to sulfide alone, but had no effect on cyanide-induced cell death (Thompson et al., 2003), suggesting that sulfide and cyanide cause cell death *via* different mechanisms. Therefore, in contrast to cyanide, which in mammalian cells does not necessarily dissipate $\Delta\Psi$m (Lawrence et al., 2001) and may not induce PT pore opening (Thompson et al., 2003), sulfide exposure of mammalian cells *in vitro* causes loss of $\Delta\Psi$m that appears to be at least partially *via* PT pore opening (Eghbal et al., 2004; Thompson et al., 2003).

It is not known whether sulfide exposure affects $\Delta\Psi$m in cells of sulfide-adapted invertebrates, and if so, whether the action is similar to that of cyanide and therefore likely occurs primarily *via* COX inhibition. This is particularly relevant, since recent evidence suggests that the mitochondria of at least some invertebrates do not respond as expected to classical PT pore inducers. Menze et al. (2005) have shown that mitochondria from the brine shrimp *Artemia franciscana*, unlike mitochondria from mammalian cells, do not swell or release the mitochondrial pro-apoptotic factor cytochrome *c* when exposed to high Ca^{2+}, although the expected molecular components of the PT pore are present (as indicated by western blot). Furthermore, Sokolova et al. (2004) found that *in vitro* Cd^{2+} exposure of hemocytes from the oyster *Crassostrea virginica* caused an apparent increase in $\Delta\Psi$m, whereas Cd^{2+} exposure of isolated oyster mitochondria caused inhibition of respiration, a slight decrease in $\Delta\Psi$m but no significant mitochondrial swelling (in contrast, PT pore opening and $\Delta\Psi$m loss are characteristic of Cd^{2+} toxicity in mammalian cells *in vitro*).

In the present study, we investigated whether sulfide exposure *in vitro* causes loss of $\Delta\Psi$m in erythrocytes from the bloodworm *Glycera dibranchiata* Ehlers 1868 (Annelida: Polychaeta: Phyllodocida: Glyceridae). To determine whether the action of sulfide was likely *via* COX inhibition, we also tested the effect of cyanide and several other mitochondrial electron transport chain inhibitors on $\Delta\Psi$m. To test whether the toxicity of sulfide was associated with free radical production, we assessed intracellular oxidative stress and mitochondrial superoxide generation during sulfide exposure. *G. dibranchiata* is abundant in fine mud with high organic content in the North Atlantic region of the USA (Wilson and Ruff, 1988), and is therefore likely to be exposed to sulfide in its natural habitat. It lacks a vascular system and its hemoglobin-containing erythrocytes, which are nucleated and contain functional mitochondria, are circulated by cilia into the thin-walled parapodia for gas exchange (Mangum, 1994), suggesting that these cells are at risk of sulfide exposure.

Materials and methods

Biological material

Glycera dibranchiata Ehlers 1868 were obtained *via* overnight delivery from a commercial supplier (Eastern Sea Worm Company, Hancock, ME, USA), which collected them by hand during low tides from mudflats of Mount Desert Island, Maine, USA. The worms were maintained in the laboratory unfed in filtered, 15°C seawater for no more than 2 weeks before being used. Immediately before an experiment, coelomic fluid was obtained from a worm through an incision in the proboscis. The fluid was then centrifuged for 30 s at 2000 ***g***, after which the supernatant and overlying white layer were discarded. The pelleted erythrocytes were then diluted 1:15 in cold incubation buffer (sterile-filtered seawater with 10 mmol l^{-1} Hepes and 0.1% glucose, pH 7.25, 1000 mOsmol kg^{-1}).

Fluorescent dyes

Live-cell fluorescent dyes were used to measure $\Delta\Psi$m, ROS and superoxide in *G. dibranchiata* erythrocytes *in vitro*. Erythrocytes (diluted up to 1:100 in incubation buffer) were loaded with dye prior to toxin exposure, except as noted below in the sulfide recovery experiment. The dye was diluted into incubation buffer from a stock solution (stored at –20°C and prepared as described below), and the erythrocytes were incubated with the dye in the dark at 15°C for 20–30 min. For each dye, loading conditions were established in preliminary experiments by confirming the appropriate labeling of cells or subcellular structures (i.e. mitochondria) using fluorescence microscopy. All dyes were from Molecular Probes, Inc. (now Invitrogen Corporation, Carlsbad, CA, USA).

JC-1 and TMRM

The cell-permeant, cationic, lipophilic fluorophores tetramethylrhodamine methyl ester (TMRM) and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) are selective for polarized mitochondria membranes under appropriate loading conditions, where they fluoresce with an intensity proportional to $\Delta\Psi$m (Bernardi et al., 1999).

Erythrocytes were loaded with JC-1 at 16 μmol l^{-1} from a 10 mmol l^{-1} stock (in dimethylsulfoxide). To reduce the presence of dye particulates prior to incubation, the JC-1 solution was sonicated for 10 min followed by centrifugation (10 min at 14 000 ***g***), with the supernatant being used for dye loading.

Erythrocytes were loaded with TMRM at 0.03–0.3 μmol l^{-1} from a 10 mmol l^{-1} stock (in ethanol). After loading, the cells were pelleted by a brief centrifugation (2 s pulse to 10 000 ***g***) and resuspended in fresh incubation buffer prior to measuring fluorescence.

H_2DCFDA and MitoSOX™ Red

Intracellular oxidative stress and free radical production were estimated using the oxidation-sensitive dyes 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) and MitoSOX™ Red mitochondrial superoxide indicator (Invitrogen Corp.), respectively. Both are nonfluorescent, cell-permeant dyes that form highly fluorescent products upon oxidization.

Oxidation of H_2DCFDA to the fluorescent product 2′,7′-dichlorofluorescein (DCF) serves as an indicator of the overall degree of intracellular oxidative stress (Barja, 2002). Erythrocytes were loaded with H_2DCFDA at 1 μg ml^{-1} from a 3 mg ml^{-1} stock solution (in dimethlysulfoxide). The cells were not rinsed by centrifugation prior to measurement of fluorescence.

Intracellular accumulation of superoxide was estimated using MitoSOX™ Red, which is selectively targeted to the mitochondria, where it is oxidized by superoxide and exhibits red fluorescence upon binding to nucleic acids. Cells were loaded with MitoSOX™ at 5 μmol l^{-1}, and were rinsed by centrifugation (as described above for TMRM) three times prior to measuring fluorescence.

Toxins

In vitro sulfide exposures were carried out in 20 ml glass vials. After loading with dye, 100–400 μl of diluted erythrocytes were added to a vial. The vial was then sealed with a rubber stopper and a volume of H_2S gas (pure or diluted 1:10 or 1:100 with room air) was added to the vial air space to bring the final H_2S composition in the gas phase to 0.1%, 0.32%, 1.0%, 3.2% or 10.0%. Pure H_2S gas was prepared fresh daily in a fume hood by reacting 0.5 g $Na_2S{\cdot}9H_2O$ with 10 ml 6 mol l^{-1} HCl in a 50 ml syringe. After injection of H_2S, the vials were placed inside a dark, 15°C incubator. After 1 h exposure, the erythrocytes were removed from the vials and placed in microcentrifuge tubes, quickly pelleted by centrifugation (a 2 s pulse to 10 000 ***g***) and resuspended in fresh incubation medium. In preliminary experiments, incubation buffer both with and without erythrocytes was assayed for total sulfide after 1 h exposure to the range of H_2S gas compositions using the Methylene Blue method (Cline, 1969). Total sulfide concentration in buffer with erythrocytes was 0.77±0.14 the sulfide concentration of buffer alone. In all experiments measuring $\Delta\Psi$m, the mitochondrial uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; 0.10 mmol l^{-1} from a 20 mmol l^{-1} stock made in ethanol and maintained at –20°C) was used as a positive control for mitochondrial membrane depolarization.

A modification of the sulfide exposure technique was conducted to determine whether the effect of sulfide was reversible after several hours. Since mitochondrial dye would tend to leak back out of the cells over a long recovery period, the erythrocytes in this experiment were loaded with dye at the end of the recovery period rather than before sulfide exposure. Erythrocytes were first exposed to sulfide for 1 h as above, and then rinsed twice by centrifugation and allowed to 'recover' at 15°C for an additional 2 or 5 h. The erythrocytes were then loaded with TMRM 30 min prior to the end of the recovery period and the fluorescence assayed as described above.

Incubations of erythrocytes with toxins other than sulfide were carried out in glass vials, as described above, or in 96-well microplates. To inhibit specific complexes of the mitochondrial electron transport chain, erythrocytes were exposed to the following toxins, for each of which the stock solutions were prepared immediately prior to the experiment: rotenone (complex I inhibitor; 10–100 μmol l^{-1} from a 20 mmol l^{-1} stock in dimethyl formamide), antimycin A (complex III inhibitor; 1–100 μmol l^{-1} from a 20 mmol l^{-1} stock in ethanol), azide (COX inhibitor; 1–10 mmol l^{-1} from a 2 mol l^{-1} N_3Na stock in incubation buffer), and cyanide (COX inhibitor; 0.1–10 mmol l^{-1} from 20 mmol l^{-1} NaCN stock in incubation buffer).

To determine whether the change in $\Delta\Psi$m from sulfide exposure is dependent on opening of the mitochondrial PT pore, *G. dibranchiata* erythrocytes were exposed to sulfide for 1 h, as described above, except that the PT pore inhibitor pair

cyclosporine A (CsA; 0.5 μmol l^{-1}) and trifluoperazine (TFP; 5 μmol l^{-1}) were added to the erythrocytes 30 min prior to initiation of sulfide exposure (Thompson et al., 2003). Control erythrocytes at each sulfide concentration were exposed to sulfide alone or sulfide with CCCP (0.10 mmol l^{-1}).

Measurement of fluorescence emission intensity

For toxin dose–response measurements using JC-1 and TMRM fluorescence emission intensity, erythrocytes that were pre-loaded with dye and had already been exposed to the toxin of interest were placed in a multi-well chamber having a #1 glass coverslip bottom (this chamber was custom-manufactured, but suitable plates are also available from Greiner Bio-One, Inc., Longwood, FL, USA). The chamber was then placed on the stage of an inverted, reflected fluorescence microscope (Olympus IX-70) and the erythrocytes were excited with light from a 100 W mercury bulb that had passed through a 90% or 99% neutral density filter (Thorlabs, Inc., Newton, NJ, USA) and a 484/15 nm (JC-1) or 555/15 nm (TMRM) excitation filter (Chroma Technology Corp, Rockingham, VT, USA). The excitation filters were positioned and shuttered using a computer-controlled filter wheel (TOFRA Inc., Palo Alto, CA, USA). Emitted light from the erythrocytes was filtered with a triple bandpass polychroic filter set (DAPI/FITC/TRITC; Chroma Technology Corp.) mounted in the standard microscope filter carousel. For quantitation of emission intensity, light from the microscope side-port was passed through a collimating lens (Thorlabs, Inc.) coupled with an SMA connector (ThorLabs, Inc.) to a 600 μm diameter fiber optic cable, and then to a linear diode-array fluorometer (SF2000, Ocean Optics Inc., Dunedin, FL, USA). Each excitation typically lasted 1 s or less. JC-1 fluorescence was quantified ratiometrically by simultaneously measuring emission intensity at 590/20 nm and 530/20 nm (i.e. red:green emission ratio), whereas TMRM fluorescence emission was measured only at 590/20 nm (i.e. red emission intensity). All fluorescence measurements were performed at 18–20°C. Digital images were acquired with a monochrome, cooled CCD camera equipped with a color LCD filter system (Retiga 2000R, QImaging Corp., Burnaby, BC, Canada).

For measurement of sulfide exposure effects over time, erythrocytes preloaded with TMRM were placed in a gas-tight Leiden chamber (Harvard Apparatus, Holliston, MA, USA) to which 10% H_2S gas was added by syringe. Emission intensity was then measured, as otherwise described above, at 0, 5, 10, 20, 30, 45 and 60 min at the same location in the well, and therefore from the same erythrocyte population.

The JC-1 frequently loaded unevenly, because even after sonicating and centrifuging the JC-1 incubation solution, minute particles of dye frequently persisted and adhered to the coverslip or the erythrocytes. Therefore, to prevent these particles from biasing the measurements, JC-1 emission intensity was measured from single erythrocytes that were pre-selected as being appropriately labeled. In this technique, single erythrocytes were placed in the center of the microscope's field of view and imaged using a 100× oil-immersion objective with additional 1.5× magnification provided by the microscope's intermediate magnification changer. The excitation light was then constrained to just outside the perimeter of the cell using the Hg lamp field iris diaphragm, and the fluorescence emission was ported to the diode array fluorometer, as described above. Despite measuring from only a single cell, integration times of 64–256 ms were typically sufficient for counts reaching between 20% and 80% of the fluorometer's dynamic range. To avoid experimenter bias in selecting appropriate erythrocytes for measurement, the order of toxin treatments on the multi-well chamber was randomized by another person; i.e. the measurements were made 'single-blind'. In contrast to JC-1, TMRM generally loaded evenly, so fluorescence emission was measured with a larger field of view (20× or 40× objective, thereby imaging up to hundreds of cells simultaneously, depending on the extent of dilution).

To confirm that effects of the sulfide on JC-1 and TMRM fluorescence were due to a change in erythrocyte $\Delta\Psi m$ rather than to direct chemical interaction of sulfide with the dye molecules, JC-1 and TMRM were each diluted in incubation buffer and exposed to control conditions (air) or 10% H_2S gas for 30 min in sealed gas vials. Each solution was then placed in a 1 cm quartz fluorescence cuvette and excited at 484/15 nm (JC-1) or 555/15 nm (TMRM) from the microscope Hg lamp *via* a fiber optic cable. The emission light was then collected normal to the excitation light with a collimating lens attached to a second fiber optic cable in a 4-way cuvette holder (Ocean Optics, Inc.), and the emission spectrum was scanned from 500–750 nm using the diode-array fluorometer. The sulfide concentration of each dye solution was tested as above to confirm that H_2S exposures achieved sulfide concentrations of at least 1 mmol l^{-1}.

Oxidation of nonfluorescent H_2DCFDA to fluorescent DCF and fluorescence of MitoSOX™ in erythrocytes exposed to sulfide were measured in black-wall, clear-bottom, fluorescence 96-well plates (Corning Inc. Life Sciences, Acton, MA, USA) with a multimode microplate reader (Synergy SIAFRT, Bio-Tek Instruments, Inc., Winooski, VT, USA) in bottom-reading mode using 485/20 nm excitation with 530/25 nm emission for H_2DCFDA, and 528/20 nm excitation with 590/35 nm emission for MitoSOX™.

Measurement of O_2 consumption

To confirm that cyanide and azide were inhibiting COX, erythrocytes were obtained from *G. dibranchiata* as described above, but the cells were diluted 5× in pre-aerated incubation buffer. The diluted erythrocytes were then placed in a 0.6 ml volume respiration chamber (Instech Laboratories, Inc., Plymouth Meeting, PA, USA) that was held at 15°C with a refrigerating, circulating water bath. The rate of decline in oxygen pressure (P_{O_2}) was then measured using a polarographic electrode until the P_{O_2} reached ca. 80% of air-saturation (i.e. ca. 17 kPa), at which point 2 μl cyanide or azide were added from stock solutions (prepared daily) using a 10 μl syringe (Hamilton Co., Reno, NV, USA) to achieve

1 mmol l^{-1}. The rate of P_{O_2} decline was subsequently measured after toxin addition until the P_{O_2} was stable or reached 50% of air-saturation (ca. 10 kPa). Because the O_2 P_{50} of *G. dibranchiata* hemolysate is 0.60 kPa (Harrington et al., 1978), unloading of O_2 at 50% air-saturation was minimal. A change in O_2 consumption rate before and after toxin addition was therefore interpreted as a toxin effect. Any apparent increase in P_{O_2} immediately after addition of toxin was attributed to a solvent artifact. Calibration of the O_2 electrode was performed daily following the manufacturer's instructions.

Statistical analyses

All data are presented as mean ± 1 S.D. (standard deviation) unless noted otherwise. In time-course measurements, emission intensity was measured over time in the same erythrocyte population, so statistical analyses of emission intensity were performed by comparing the overall emission at each time point with the emission at the initial time point (time=0) using repeated-measures analysis of variance (RM ANOVA). In all measurements of toxin dose–response, fluorescence emission was determined for at least 10 cells at each concentration for JC-1, or at four non-overlapping positions in a well for TMRM. These values were subsequently averaged to give the mean emission ratio (JC-1) or mean emission (TMRM) for the toxin concentration in that well. The data could be tested by RM ANOVA because diluted erythrocytes from one animal were used for all toxin concentrations within a trial, as well as for the negative control (no toxin) and, in the case of $\Delta\Psi$m measurements, for the positive control (CCCP) for that trial. For the sulfide recovery experiment, significant changes in emission intensity and interactions between factors (time and concentration) were detected by two-factor RM ANOVA. For all RM ANOVA analyses, replicates represent separate experiments conducted with erythrocytes from different worms. All RM ANOVA tests were performed on raw data (fluorescence counts), but for graphical presentation of these data in the figures, the values at each treatment level (i.e. toxin concentration) were divided by (i.e. 'normalized' to) the initial value (for time-series measurements) or the negative control value (for dose–response measurements) for that erythrocyte sample (i.e. for erythrocytes from a given animal). *Post-hoc* multiple comparisons for all ANOVA tests were performed with the Tukey HSD procedure. Statistical analyses were conducted using Statistica 5.5 (StatSoft, Inc., Tulsa, OK, USA), with $P<0.05$ accepted as significant.

For sulfide dose–response measurements, the change in fluorescence emission (or emission ratio) with increasing sulfide concentration was fit to the standard sigmoidal dose–response curve $y=a+[(1-a)/(1+10^{(\log EC_{50}-x)n}]$, where a is the minimum relative fluorescence emission, EC_{50} is the concentration of toxin at which TMRM fluorescence is decreased by 50% relative to the control, x is $\log_{10}$ of the toxin concentration, and n is the Hill coefficient. Data for the curve fitting were normalized to the control value for each erythrocyte population, and were fit using the non-linear curve-fitting function of SigmaPlot 9.01 (Systat Software, Inc., Point Richmond, CA, USA).

Results

Labeling of erythrocytes by JC-1 and TMRM

Under control conditions, *G. dibranchiata* erythrocytes appeared pink with an uneven surface under brightfield microscopy (Fig. 1A,G). With fluorescence microscopy, erythrocytes loaded with JC-1 demonstrated punctate red-orange fluorescence (Fig. 1D), and erythrocytes loaded with TMRM exhibited punctuate red fluorescence (Fig. 1J; note that the cells in Fig. 1J,K,L are counterstained with the nuclear dye Hoechst 33342). Therefore, both JC-1 and TMRM showed punctate staining consistent with specific labeling of mitochondria. Exposure of erythrocytes to 10% H_2S gas (yielding ca. 1.9 mmol l^{-1} sulfide) for 1 h caused the surface of the erythrocytes to become smoother (Fig. 1B,H), and caused a shift in JC-1 fluorescence from punctate red–orange to diffuse green (Fig. 1E). TMRM fluorescence was also

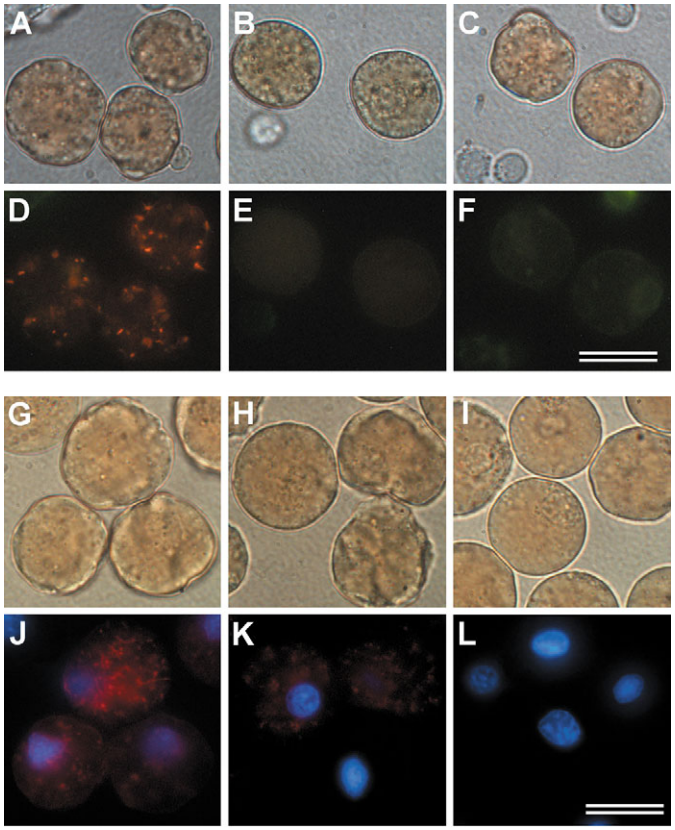

Fig. 1. Light micrographs and fluorescence micrographs of *G. dibranchiata* erythrocytes labeled with JC-1 (A–F) and TMRM (G–L) and exposed to control conditions (A,D,G,J), 1.9 mmol l^{-1} sulfide (B,E,H,K), or 0.10 mmol l^{-1} CCCP (C,F,I,L). Erythrocytes labeled with JC-1 were imaged in real color using a triple-bandpass filter set, as described in the text. Erythrocytes labeled with TMRM were counterstained with the nuclear dye Hoechst 33342, after which each dye was imaged in monochrome and pseudocolored for this figure. Scale bars, 10 μm.

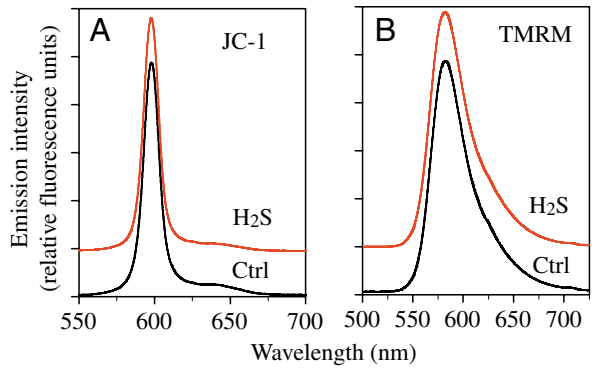

Fig. 2. Emission spectra of TMRM (A) and JC-1 (B) in sulfide-free incubation buffer (black, lower line) or in incubation buffer with 1.9 mmol l^{-1} sulfide (red, upper line). Note that the green fluorescence of the monomeric form of JC-1 did not contribute significantly to JC-1 emission spectra in this preparation.

decreased by sulfide exposure, although it did not disappear (Fig. 1K), and unlike JC-1, TMRM showed no evidence of an increase in fluorescence at a shorter wavelength. Exposure of erythrocytes to the mitochondrial uncoupler CCCP (0.10 mmol l^{-1}) for 1 h generally also resulted in a smoother, rounder appearance under brightfield microscopy (Fig. 1C,I). Under fluorescence microscopy, erythrocytes labeled with JC-1 showed diffuse green fluorescence with very little red fluorescence (Fig. 1F), comparable to sulfide exposure. Similarly, erythrocytes labeled with TMRM showed an almost complete loss of red fluorescence (Fig. 1L). Therefore, both JC-1 and TMRM showed a change in fluorescence emission after sulfide exposure consistent with loss of $\Delta\Psi$m.

To test whether sulfide reacted chemically with JC-1 or TMRM to cause the observed fluorescence emission changes, both dyes were exposed to sulfide under cell-free conditions and the fluorescence spectra were recorded. Under control conditions, both dyes exhibited typical fluorescence spectra: when excited at 484 nm, JC-1 had a peak emission of 597 nm (Fig. 2A, black line), consistent with the expected peak emission of 595 nm (spectral data from Invitrogen Corp.), whereas TMRM had peak emission at 582 nm when excited at 530 nm (Fig. 1B, black line), similar to the expected value of 576 nm (based on spectral data for the related dye TAMRA, from Invitrogen Corp.). When the dye solutions were exposed to 10% H_2S gas for 30 min in sealed vials, the emission spectra showed no detectable change from the control spectra (red lines in Fig. 2A,B; note that these lines are arbitrarily offset to allow comparison with the control spectra). Therefore, neither JC-1 nor TMRM are affected by ca. 1.9 mmol l^{-1} sulfide under cell-free conditions.

Although we found that both JC-1 and TMRM labeled mitochondria in *G. dibranchiata* erythrocytes, each dye had specific advantages and disadvantages. JC-1 staining solution was more difficult to prepare, primarily owing to its low solubility in aqueous medium and tendency to form particulates that were difficult to remove even with prolonged centrifugation. This necessitated measuring the fluorescence of erythrocytes individually in the modified fluorescence microscope, which proved quite time-consuming (note that a flow cytometer would likely be a useful alternative in this case). However, an advantage of JC-1 was its specificity; it had little tendency to label cellular structures other than mitochondria. Nonetheless, for reasons that were not clear, JC-1 sometimes failed to label any structures. TMRM, in contrast, while simple and inexpensive to prepare, had a tendency to label non-mitochondrial structures (presumably endoplasmic reticulum or nuclear membranes), especially at higher dye concentrations, necessitating careful attention to loading conditions and confirmation of specificity by fluorescence microscopy before each experiment. Another difference is that while JC-1 fluorescence is measured at two emission wavelengths ratiometrically, TMRM fluorescence is typically measured at a single wavelength (Bernardi et al., 1999), although note that TMRM emission can also be measured at two excitation wavelengths, which has been reported to provide increased sensitivity (Scaduto and Grotyohann, 1999) and has been used for measuring $\Delta\Psi$m in oyster hemocytes (Sokolova et al., 2004). In our hands, JC-1 and TMRM had similar sensitivity and signal-to-noise ratio (data not shown). On balance, TMRM proved the most practical, and therefore after validating the effect of sulfide on $\Delta\Psi$m in erythrocytes (below), the data for the remaining experiments were with TMRM.

Sulfide-$\Delta\Psi$m dose–response

Exposure of *G. dibranchiata* erythrocytes loaded with JC-1 to sulfide concentrations of 110 μmol l^{-1} to 1.9 mmol l^{-1} for 1 h produced a significant decrease in the JC-1 fluorescence ratio (Fig. 3A; N=8, RM ANOVA $F_{8,64}$=11, P<0.0001), consistent with a dose-dependent decrease in $\Delta\Psi$m, with a significant change from control fluorescence first evident at 0.73 mmol l^{-1} (P=0.00052). The data fitted well with a sigmoidal dose–response curve (r^2=0.49, P<0.0001), with an apparent 50% maximal effect (EC_{50}) at 0.50 mmol l^{-1} sulfide. Exposure to CCCP (0.10 mmol l^{-1}) for 1 h also produced a decrease in the JC-1 fluorescence that was similar in magnitude to the decrease recorded with the maximum sulfide concentration ratio (Fig. 3B; N=8, one-tail paired t-test t_7=5.4, P=0.00049). Erythrocytes loaded with TMRM showed a similar dose-dependent decrease in fluorescence that was characteristic of a decrease in $\Delta\Psi$m (Fig. 3C; N=4, RM ANOVA $F_{7,21}$=6.7, P<0.00030), with a significant change from control fluorescence first evident at 1.1 mmol l^{-1} (P=0.0050). As with JC-1, the TMRM response fitted well with a sigmoidal dose–response curve (r^2=0.63, P<0.0001), with the apparent EC_{50} at 0.83 mmol l^{-1} sulfide. Exposure to CCCP for 1 h produced a decrease in fluorescence that was similar to that of the highest sulfide concentration (Fig. 3D; N=4, one-tail paired t-test t_3=3.8, P=0.016).

Time-course of $\Delta\Psi$m during sulfide exposure

When *G. dibranchiata* erythrocytes were loaded with TMRM and then incubated under control conditions for 1 h,

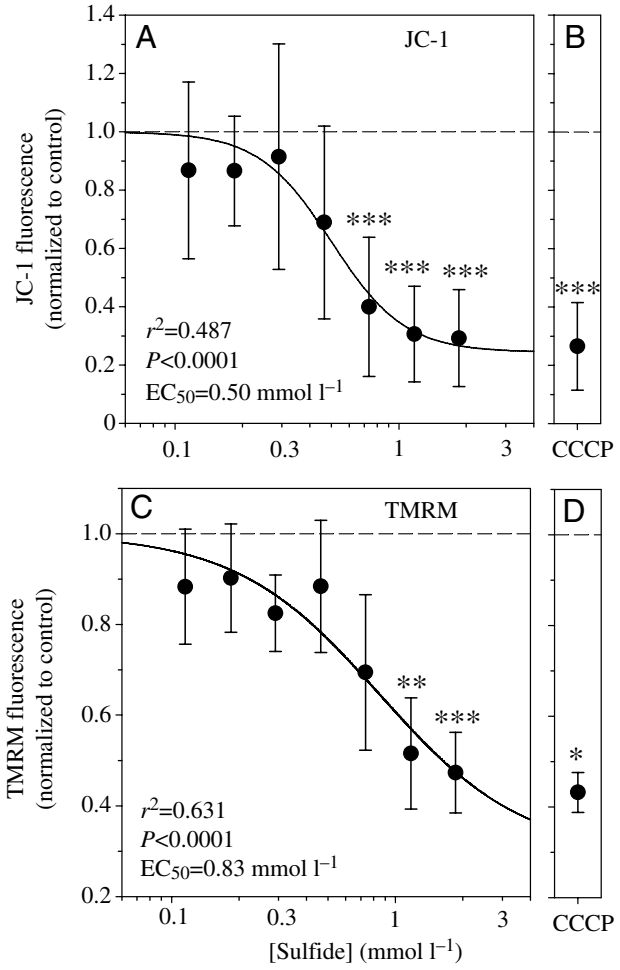

Fig. 3. Fluorescence of JC-1 (A,B) and TMRM (C,D) in *G. dibranchiata* erythrocytes exposed to sulfide (A,C; 0.11–1.9 mmol l^{-1}) or CCCP (B,D; 0.10 mmol l^{-1}) for 1 h. JC-1 fluorescence ratio and TMRM fluorescence emission at each sulfide concentration are normalized to that of control erythrocytes (erythrocytes incubated in sulfide-free buffer for 1 h). Values are means ± S.D. (N=8 for JC-1, N=4 for TMRM). CCCP (0.10 mmol l^{-1}) was added as a positive control for loss of $\Delta\Psi$m. The regression line represents a sigmoidal dose–response curve (see equation in text) with correlation coefficient, significance and EC_{50} listed in each panel. Asterisks represent a significant effect of sulfide or CCCP compared to the control value (no toxin) by RM ANOVA (*P<0.05, **P<0.01, ***P<0.005).

the fluorescence intensity did not change (Fig. 4, open symbols; N=5, RM ANOVA $F_{5,20}$=0.78, P=0.57), consistent with maintenance of a stable $\Delta\Psi$m over the measurement period. However, exposure to 1.9 mmol l^{-1} sulfide (10% H_2S gas) for 1 h produced a significant decrease in fluorescence (Fig. 4, closed symbols; N=5, RM ANOVA $F_{5,20}$=7.72, P=0.00035), consistent with loss of $\Delta\Psi$m.

Recovery from sulfide exposure

To determine whether the effect of sulfide on $\Delta\Psi$m is transient and reversible, *G. dibranchiata* erythrocytes were exposed to a range of sulfide concentrations for 1 h, rinsed twice in sulfide-free incubation buffer and then allowed to recover for 2 or 5 h (3 and 6 h experiment duration, respectively). Unlike the dose–response experiments (described in the previous section), erythrocytes in this recovery experiment were not loaded with TMRM until the last 30 min of the recovery period. Therefore, given that H_2S volatilization and sulfide oxidation should have rapidly eliminated any free sulfide, it is reasonable to assume that mitochondrial TMRM incorporation at 2 and 5 h recovery occurred under sulfide-free conditions. After exposure to 0.3, 0.5, 0.8 and 1.2 mmol l^{-1} sulfide for 1 h, there was a significant decrease in TMRM fluorescence due to sulfide, but no effect of time and no interaction between these factors (Fig. 5; N=5, two-factor RM ANOVA; sulfide main effect $F_{4,8}$=33, P<0.0001; time main effect $F_{2,4}$=0.20, P=0.83; sulfide×time effect $F_{8,16}$=0.44, P=0.88). Immediately following 1 h sulfide exposure, TMRM fluorescence was significantly decreased at all sulfide concentrations compared to the control erythrocytes, as expected. After 2 h recovery, only the erythrocytes that had been exposed to the highest sulfide concentration were still significantly different from the control, whereas after 5 h recovery, erythrocytes exposed to all but the lowest sulfide concentration were significantly below the control erythrocytes.

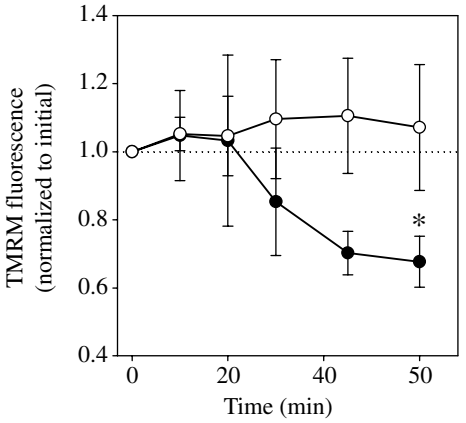

Fig. 4. Time course of TMRM fluorescence from *G. dibranchiata* erythrocytes exposed to control conditions (open symbols) or to 1.9 mmol l^{-1} sulfide (closed symbols) for 1 h. Fluorescence emission (595 nm) at each time point is normalized to the initial value (t=0) for each treatment. Values are means ± S.D. (N=5). Asterisks represent a significant effect over time compared to the initial value by RM ANOVA (*P<0.05).

Effect of ETC inhibitors

G. dibranchiata erythrocytes were exposed to other mitochondrial electron transport chain (ETC) inhibitors to determine whether the effect of sulfide on $\Delta\Psi$m is consistent with its known inhibitory effect on COX (complex IV). Erythrocytes were loaded with TMRM and exposed to rotenone (complex I inhibitor), antimycin A (complex III inhibitor), and the COX inhibitors cyanide and azide (Fig. 6). Rotenone produced a significant decrease in TMRM

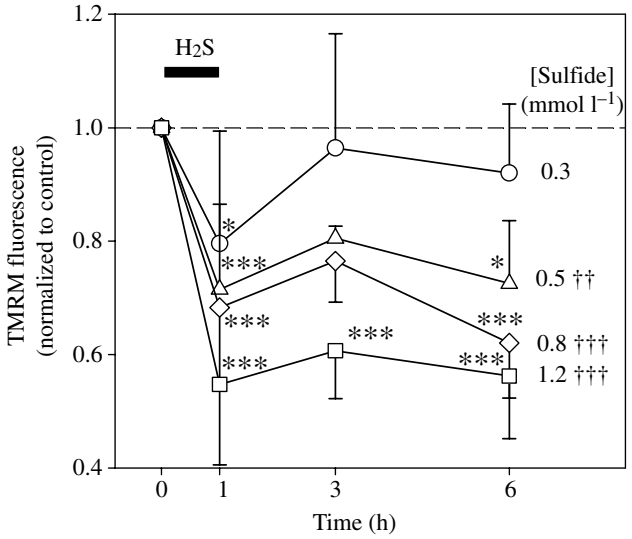

Fig. 5. TMRM fluorescence in *G. dibranchiata* erythrocytes exposed to sulfide (0.3, 0.5, 0.8 and 1.2 mmol l^{-1}) for 1 h followed by recovery in sulfide-free buffer for 2 or 5 h. Fluorescence emission (595 nm) at each time point is normalized to the emission of control erythrocytes (sulfide-free buffer) at the same time point. Values are means ± S.D. (*N*=3). Statistical analyses were performed by two-factor, RM ANOVA followed by Fisher's LSD *post-hoc* test (note that this is less conservative than the Tukey HSD test). Asterisks adjacent to data points indicate a significant difference from the control value (no sulfide) at the same time point (**P*<0.05, ***P*<0.01, ****P*<0.005); daggers indicate a significant main effect of sulfide at that concentration ($^{\dagger}P$<0.05, $^{\dagger\dagger}P$<0.01, $^{\dagger\dagger\dagger}P$<0.005). Note that there was no significant effect of time (*P*=0.83) and no significant interaction between sulfide and time (*P*=0.88).

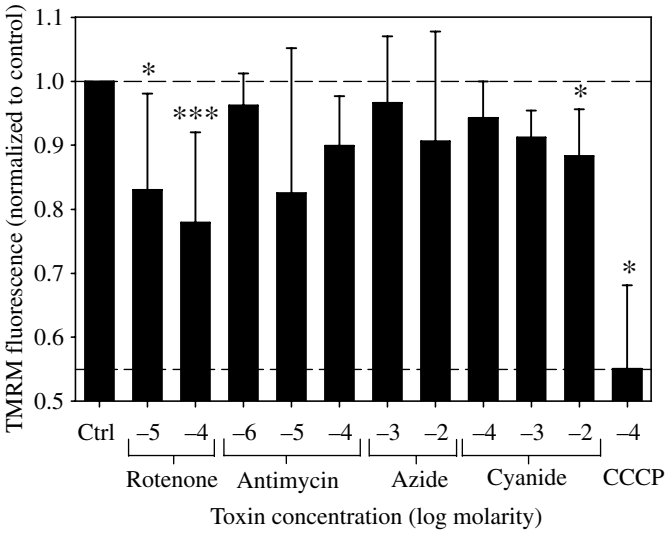

Fig. 6. Fluorescence of TMRM in *G. dibranchiata* erythrocytes exposed to the mitochondrial electron transport chain inhibitors rotenone (10 and 100 μmol l^{-1}, *N*=10), antimycin (1, 10 and 100 μmol l^{-1}, *N*=10, 10 and 4, respectively), azide (1 and 10 mmol l^{-1}, *N*=10), and cyanide (0.1, 1 and 10 mmol l^{-1}, *N*=4). CCCP (0.10 mmol l^{-1}, *N*=10) was added as a positive control for loss of ΔΨm. Fluorescence emission (595 nm) for each inhibitor is normalized to control conditions (buffer only). Values are means ± S.D. Asterisks represent a significant effect of toxin or CCCP compared to the control value (no toxin) by RM ANOVA (**P*<0.05, ****P*<0.005).

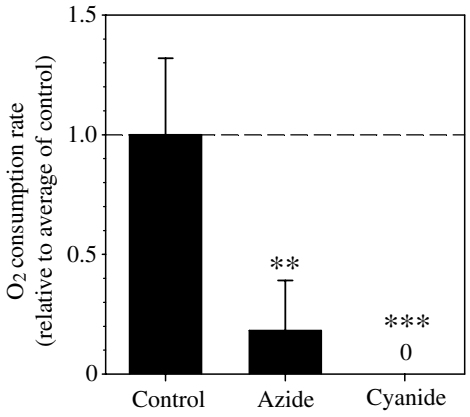

Fig. 7. O_2 consumption of *G. dibranchiata* erythrocytes under control conditions or exposed to the mitochondrial COX inhibitors sodium azide (1 mmol l^{-1}) and sodium cyanide (1 mmol l^{-1}). Values are means ± S.D. (*N*=5 for CN, *N*=3 for azide). O_2 consumption is presented relative to the average at control conditions (i.e. consumption rate immediately prior to addition of inhibitor). Asterisks represent a significant decrease in consumption rate to the control value (no toxin) by one-tailed, paired *t*-test (***P*<0.01, ****P*<0.005).

fluorescence (*N*=10, RM ANOVA, $F_{2,18}$=7.7, *P*=0.0038), but no significant change in fluorescence was seen after exposure to antimycin (*N*=4–10, $F_{3,9}$=2.7, *P*=0.11) or azide (*N*=10, $F_{2,18}$=1.1, *P*=0.34). Cyanide also had no effect, except at the highest concentration of 10 mmol l^{-1} (*N*=4, $F_{3,9}$=5.6, *P*=0.019), and even then the effect was small. CCCP served as a positive control for each experiment and showed the expected decrease in fluorescence (*N*=10, one-tailed, paired *t*-test t_9=2.6, *P*=0.014).

To confirm that the absence of a large change in ΔΨm with azide and cyanide was not due to lack of COX inhibition, the effect of these toxins on whole-cell O_2 consumption was measured. *G. dibranchiata* erythrocytes were exposed to 1 mmol l^{-1} azide or 1 mmol l^{-1} cyanide. Both toxins showed a large decrease in O_2 consumption when compared with control conditions, which was significant by one-tailed, paired *t*-test (Fig. 7: azide, *N*=3, t_2=9.8, *P*=0.0051; cyanide, *N*=5, t_4=7.5, *P*=0.00083).

PT pore inhibitors

The PT pore inhibitors CsA (0.5 μmol l^{-1}) and TFP (5 μmol l^{-1}) were added to *G. dibranchiata* erythrocytes prior to sulfide exposure to test whether the decrease in ΔΨm during sulfide exposure was at least partially dependent on PT pore opening. As expected, sulfide alone caused a decrease in TMRM fluorescence (Fig. 8, circles, *N*=5, $F_{3,9}$=8.0, *P*=0.0067), but the addition of CsA and TFP with sulfide exposure did not affect TMRM fluorescence compared to

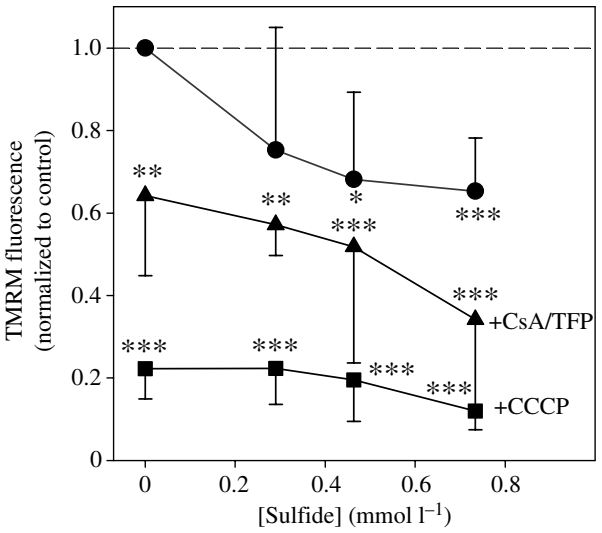

Fig. 8. TMRM fluorescence in *G. dibranchiata* coelomocytes exposed for 1 h to various sulfide concentrations (up to 0.73 mmol l^{-1}) either alone (circles) or with CCCP (0.10 mmol l^{-1}; squares) or PTP inhibitors (0.5 μmmol l^{-1} CsA and 5 μmol l^{-1} TFP; triangles). Fluorescence emission (595 nm) is normalized to control conditions (1 h exposure to buffer alone). Values are means ± S.D. (N=5). Asterisks represent a significant effect of sulfide, CCCP or CsA/TFP compared to the control value by two-factor, RM ANOVA (*P<0.05, **P<0.01, ***P<0.005). Refer to text for further results of statistical analyses.

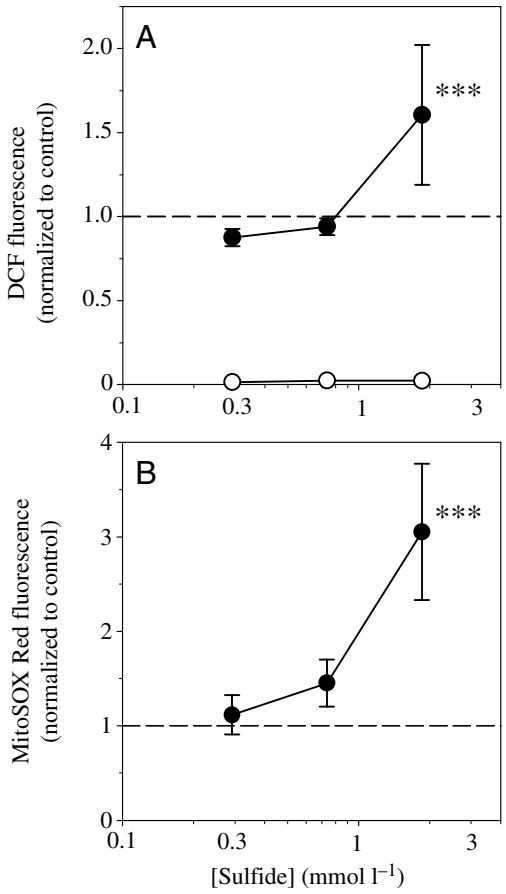

Fig. 9. Enhanced production of reactive oxygen species in *G. dibranchiata* erythrocytes exposed to sulfide (up to 1.9 mmol l^{-1}) for 1 h. (A) Formation of fluorescent 2′,7′-dichlorofluorescein (DCF) from nonfluorescent 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) in erythrocytes (closed circles) or incubation buffer alone (open circles). DCF fluorescence is normalized to DCF formation in erythrocytes under control conditions (no sulfide). (B) Oxidation of MitoSOX™ Red in erythrocytes. Fluorescence is normalized to control conditions (no sulfide). Values are means ± S.D. (N=4). Asterisks represent a significant effect of sulfide compared to the control value (no sulfide) by RM ANOVA (***P<0.005).

sulfide alone (Fig. 8, triangles, N=4–5, P=0.087). In fact, addition of CsA and TFP caused decreased fluorescence even in the absence of sulfide (P=0.010). CCCP caused a decrease in fluorescence in the absence of sulfide (P=0.00016), as expected, but TMRM fluorescence in erythrocytes exposed to the combination of 0.73 mmol l^{-1} sulfide with CCCP was significantly lower than fluorescence in erythrocytes exposed to CCCP alone (i.e. there was an additive effect of sulfide on CCCP; Fig. 8, squares, $F_{3,12}$=13, P=0.00041).

Reactive oxygen species

To determine whether significant production of ROS occurs during sulfide exposure, *G. dibranchiata* erythrocytes were loaded with the oxidative stress-sensitive dye H_2DCFDA and the mitochondria-specific, superoxide-sensitive dye MitoSOX™ Red. Erythrocytes loaded with H_2DCFDA and exposed to 290 μmol l^{-1}, 730 μmol l^{-1} and 1.9 mmol l^{-1} sulfide for 1 h showed a 60% increase in DCF fluorescence at the highest sulfide concentration, indicating oxidation of H_2DCFDA (Fig. 9A, closed circles; N=4, RM ANOVA $F_{5,15}$=12, P<0.0001). There was no effect at the lower sulfide concentrations. Sulfide added to H_2DCFDA in incubation medium produced a significant increase in fluorescence at all sulfide concentrations, indicating some production of reactive oxygen species from spontaneous sulfide oxidation (Fig. 9A, open circles, N=6, $F_{3,21}$=15, P<0.0001), but the fluorescence intensity was 1/50th of that seen with the same concentration of H_2DCFDA loaded into erythrocytes. Similarly to H_2DCFDA, erythrocytes loaded with MitoSOX™ Red and exposed to 290 μmol l^{-1}, 730 μmol l^{-1} and 1.9 mmol l^{-1} sulfide for 1 h showed a 300% increase in fluorescence at the highest sulfide concentration (Fig. 9B, N=4, RM ANOVA $F_{3,9}$=22, P=0.00019), but had showed no significant change at the lower sulfide concentrations (P>0.44).

Discussion

Mitochondrial membrane depolarization

The primary focus of this study was the effect of sulfide exposure on ΔΨm in *G. dibranchiata* erythrocytes. As the first step, we established that erythrocytes incubated with the membrane potential-sensitive dyes JC-1 or TMRM, under the appropriate loading conditions, show fluorescence consistent

with mitochondrial labeling (Fig. 1). For both dyes, the fluorescence was stable over at least 1 h under control conditions, whereas exposure to the mitochondrial uncoupler CCCP for 1 h caused a decrease in the red:green ratio for JC-1 and a decrease in red emission intensity for TMRM, both of which are characteristic of mitochondrial membrane depolarization. Therefore, the patterns of labeling and the responses to CCCP suggest that both JC-1 and TMRM can be used as indicators of $\Delta\Psi$m in *G. dibranchiata* erythrocytes. Previous usage of mitochondrial membrane potential-sensitive dyes in invertebrate macrofauna is sparse, but TMRM has recently been shown to have a similar sensitivity to CCCP in oyster hemocytes (Sokolova et al., 2004), and JC-1 labels mitochondria and responds appropriately to an uncoupler (the K^+ ionophore valinomycin) in coelomocytes from the earthworm *Eisenia foetida* (Cossarizza et al., 1995). Rhodamine 123, which is structurally similar to TMRM, labels mitochondria and responds to CCCP appropriately in crayfish muscle (Nguyen et al., 1997) and labels mitochondria in *Drosophila melanogaster* embryos (Akiyama and Okada, 1992), although whether the fluorescence was affected by uncouplers in that case was not tested.

When *G. dibranchiata* erythrocytes loaded with JC-1 or TMRM were exposed to 1.9 mmol l^{-1} sulfide for 1 h, the fluorescence emission of both dyes changed in a manner similar to that seen after exposure of erythrocytes to CCCP (i.e. decreased red:green ratio with JC-1 and decreased red intensity with TMRM). This suggested that sulfide caused a loss of $\Delta\Psi$m. However, an alternative explanation for the change in fluorescence would be that sulfide (whether as H_2S, HS^- or S^{2-}) interacted directly with each dye to cause decreased red fluorescence. However, two lines of evidence suggest that this is not likely. First, when JC-1 and TMRM were dissolved in incubation buffer (without erythrocytes) and exposed to 1 mmol l^{-1} sulfide for 30 min, the fluorescence emission spectra of both dyes were indistinguishable from their spectra obtained prior to sulfide exposure (Fig. 2). Second, JC-1 and TMRM were specifically selected for this study because they differ substantially in chemical structure and action, even though they are both lipophilic cations that accumulate in mitochondria with high $\Delta\Psi$m. One consequence is that JC-1 exists as a green-fluorescent monomer in cells having mitochondria with low $\Delta\Psi$m, but forms red-fluorescent 'J-aggregates' upon concentration into mitochondrial membranes with high $\Delta\Psi$m (Smiley et al., 1991), whereas TMRM remains as a monomer in membranes with high $\Delta\Psi$m but exhibits a red-shift in the fluorescence emission spectrum (Scaduto and Grotyohann, 1999). Therefore, we consider it unlikely that both dyes interact directly with sulfide to cause fluorescence changes mimicking loss of $\Delta\Psi$m.

After exposure to a range of sulfide concentrations for 1 h, *G. dibranchiata* erythrocytes loaded with JC-1 or TMRM exhibited a concentration-dependent change in fluorescence consistent with decreased $\Delta\Psi$m (Fig. 3). Furthermore, the magnitude of the fluorescence change at sulfide concentrations of 1.2 mmol l^{-1} and higher was similar to that of CCCP, suggesting that sulfide induced mitochondrial depolarization to an extent equivalent to pharmacological uncoupling. Similarly, Eghbal et al. (2004) used rhodamine 123 incorporation (rather than fluorescence change) to show that isolated rat hepatocytes exposed to 0.5 mmol l^{-1} sulfide for 1 h decreased $\Delta\Psi$m by 50%. Using CCCP-induced depolarization as a reference point, we found a comparable sensitivity in *G. dibranchiata* erythrocytes, in which the sulfide EC_{50} for $\Delta\Psi$m was 0.50–0.83 mmol l^{-1} (using JC-1 and TMRM, respectively), suggesting a surprisingly similar sulfide cytotoxicity for rats and *G. dibranchiata*.

Although inhibition of COX by sulfide is reversible *in vitro* (Nicholls and Kim, 1982) and probably *in vivo* (Blackstone et al., 2005), the loss of $\Delta\Psi$m in *G. dibranchiata* erythrocytes following 1 h sulfide exposure *in vitro* was irreversible over a subsequent 5 h recovery period (Fig. 5). This implied that the toxic effect of sulfide was not simply due to inhibition of COX. We further tested this by exposing erythrocytes to four other mitochondrial electron transport chain inhibitors: rotenone, antimycin, azide and cyanide. Of these, only rotenone and cyanide caused a significant change in $\Delta\Psi$m, but for neither of these inhibitors was the degree of depolarization comparable to that achieved with CCCP or sulfide (Fig. 6). That 10 mmol l^{-1} azide and cyanide had either no effect or caused only a 20% decrease in $\Delta\Psi$m (in comparison to CCCP) was surprising since, like sulfide, both are COX inhibitors, and both caused an immediate, substantial reduction in O_2 consumption even when applied at only 1 mmol l^{-1} concentration (Fig. 7). Taken together, the irreversibility of sulfide and the absence of a similar effect from other COX inhibitors provide strong evidence that the mechanism by which sulfide causes mitochondrial depolarization is not *via* COX inhibition. A similar conclusion was reached by Thompson et al. (2003) who, like Eghbal et al. (2004), studied the effect of sulfide on primary rat hepatocytes in culture. Thompson et al. (2003) found that hepatocytes exposed to sulfide or cyanide were killed in a dose-dependent manner, as expected, but differed in their response to supplementation by glycolytic substrate. Specifically, addition of fructose to the hepatocytes, which greatly enhances glycolytic ATP production in these cells (Nieminen et al., 1994), substantially decreased the toxicity of cyanide, but had no effect on the toxicity of sulfide, and the PT pore inhibitors CsA and TFP decreased cell death during sulfide exposure but not during cyanide exposure (Thompson et al., 2003). It should noted, however, that since the response of hepatocytes to fructose supplementation is not a characteristic of other mammalian cells, the reduced toxicity of cyanide, but not sulfide, with added fructose may be unique to liver.

Mitochondrial membrane permeability

Sulfide-induced, irreversible opening of the PT pore would be consistent with the loss of $\Delta\Psi$m seen in *G. dibranchiata* erythrocytes. In support of this, the PT pore inhibitors CsA and TFP decreased cell death by up to 50% in rat hepatocytes exposed to sulfide (Thompson et al., 2003). However, in the

present study, addition of CsA and TFP did not prevent loss of $\Delta\Psi$m during sulfide exposure (Fig. 8). Thus, CsA and TFP together reduce sulfide cytotoxicity but not sulfide-induced loss of $\Delta\Psi$m. Reconciling these findings requires that (1) sulfide-induced loss of $\Delta\Psi$m does not necessarily lead to erythrocyte death and (2) at least some sulfide-induced cell death is *via* a mechanism that is inhibited by CsA and TFP but does not result in maintenance of $\Delta\Psi$m. Although this has not been tested with sulfide, loss of $\Delta\Psi$m can result from many events other than PT pore opening in mammalian cells, including increased ATP demand and uncoupling of oxidative phosphorylation (Bernardi et al., 2001; Green and Kroemer, 2004; Ly et al., 2003), and cell death is not necessarily a consequence if $\Delta\Psi$m loss is transient (Ly et al., 2003). With regard to an alternative protective role of CsA or TFP, in addition to its effect on the PT pore, CsA inhibits all cyclophilins and also has recently been shown to inhibit Ca^{2+} uptake by mitochondria through the Ca^{2+} uniporter (Montero et al., 2003). Furthermore, release of pro-apoptotic proteins from mitochondria requires increased permeability of the mitochondrial outer membrane, whereas the PT pore spans the inner membrane. The apparent mechanistic link is that PT pore opening with the subsequent influx of ions into the mitochondrial matrix promotes the osmosis of sufficient water into the mitochondrial matrix to rupture the outer membrane (Green and Kroemer, 2004; Halestrap et al., 2002). Therefore, it is worth noting that neither sulfide nor cyanide caused swelling in mitochondria from rat hepatocytes (Thompson et al., 2003), suggesting that neither caused PT pore opening.

Nonetheless, this does not rule out PT pore opening in sulfide-exposed erythrocytes. The structure of the PT pore is controversial, but it appears to be a complex with three major components: a voltage-dependent anion channel (VDAC) from the outer membrane, an adenine nucleotide translocator (ANT) from the inner membrane, and cyclophilin D (CyP-D), which binds to ANT and promotes PT pore opening (Green and Kroemer, 2004; Halestrap et al., 2002; Kim et al., 2003a; Ly et al., 2003). CsA normally inhibits PT pore opening by binding to CyP-D, but pore opening is insensitive to CsA at high concentrations of various inducers (Halestrap et al., 2002; He and Lemasters, 2002), suggesting that sulfide could initiate PT pore opening even in the presence of CsA. Furthermore, TFP is not a potent inhibitor of PT pore opening under de-energized conditions (Halestrap et al., 1997), and therefore may not protect cells from sulfide exposure. Finally, the ANT has three cysteine residues that appear to regulate CyP-D activity and that can be modified by thiol reagents and reactive oxygen species (Halestrap et al., 1997). Unlike cyanide or azide, sulfide is a highly reactive thiol compound that may be capable of directly modifying one or more of these residues, potentially inducing pore opening. Finally, an additional possibility is that sulfide induces opening of an unregulated PT pore in *G. dibranchiata* erythrocytes. The presence of an unregulated pore, which is not inhibited by CsA (He and Lemasters, 2002; Menze et al., 2005), has been proposed in the crustacean *A. franciscana* (Menze et al., 2005).

ROS production and oxidative stress

Increased oxidative stress is an additional mechanism by which sulfide exposure could cause toxicity (Abele-Oeschger, 1996; Abele-Oeschger et al., 1994; Morrill et al., 1988), and therefore mitochondrial depolarization. Sulfide oxidizes spontaneously in the presence of divalent metals (both dissolved and in metalloenzymes), generating oxygen-centered and sulfur-centered radicals in aqueous solutions (Chen and Morris, 1972; Tapley et al., 1999) and in animal tissues (Tapley, 1993). Recently, Eghbal et al. (2004) used the ROS indicator H_2DCFDA in rat hepatocytes and showed that free radical-induced oxidation of this dye to DCF was 2–3 times faster when the cells were exposed to 0.5 mmol l^{-1} sulfide than when they were exposed to cyanide or control conditions. Furthermore, the addition of ROS scavengers decreased cell death by up to 40% in hepatocytes exposed to 0.5 mmol l^{-1} sulfide for 3 h, although this protective effect was not seen after 1 h (Eghbal et al., 2004). In this study, we found that *G. dibranchiata* erythrocytes loaded with H_2DCFDA and exposed to sulfide for 1 h showed a 60% increase in DCF fluorescence at the highest sulfide concentration (1.9 mmol l^{-1}), indicating oxidation of H_2DCFDA (Fig. 9A). This effect was not seen at 0.73 mmol l^{-1} and lower sulfide concentrations. Increased H_2DCFDA oxidation was not due to spontaneous sulfide oxidation, since H_2DCFDA in cell-free incubation medium oxidized to DCF at less than 2% of the rate seen in medium with erythrocytes. Therefore, the effect of sulfide on H_2DCFDA in *G. dibranchiata* erythrocytes is comparable to its effect in rat hepatocytes, although in *G. dibranchiata* the effect is right-shifted with respect to sulfide concentration. Because superoxide may be a reaction product in the first sulfide oxidation step (Chen and Morris, 1972; Tapley et al., 1999), we specifically investigated whether sulfide exposure increases superoxide generation. We utilized the dye MitoSOX™ Red, which is taken up by mitochondria and is readily oxidized by superoxide but not by other ROS or reactive nitrogen species (data from Invitrogen Corp.). Similar to the results with oxidative stress indicator H_2DCFDA, we found that MitoSOX™ oxidation was increased threefold in *G. dibranchiata* erythrocytes exposed to 1.9 mmol l^{-1} sulfide for 1 h, but not by exposure to 0.73 mmol l^{-1} or lower sulfide concentrations (Fig. 9B). In addition to sulfide oxidation as a source, superoxide is also generated within mitochondria as a byproduct of oxidative phosphorylation (Halliwell and Gutteridge, 1999), and the present study does not distinguish between these sources. Theoretically, increased mitochondrial ROS production may also occur specifically from periodic exposure to sulfide (such as with tidal cycles for mudflat animals), because the resultant periodic COX inhibition might increase mitochondrial free-radical production by a process essentially identical to that of hypoxia/reoxygenation injury (Kim et al., 2003b). However, whether this occurs following sulfide exposure has not been investigated.

While increased oxidation of H_2DCFDA will result from augmented ROS production, DCF production may also be enhanced by depletion of intracellular antioxidants. For this reason, the rate of DCF production is best interpreted as an estimator of the overall degree of oxidative stress within cells (Barja, 2002), with the same presumably also applying to MitoSOX^{TM}. Therefore, since significant H_2DCFDA oxidation occurred at lower sulfide concentrations in rat hepatocytes than in *G. dibranchiata* erythrocytes, this suggests that either *G. dibranchiata* erythrocytes have increased antioxidant defenses compared to rat hepatocytes, or that rat hepatocytes produce more free radicals than *G. dibranchiata* erythrocytes at a given sulfide concentration. In fact, both *G. dibranchiata* erythrocytes and rat hepatocytes may be particularly susceptible to oxidative damage from sulfide exposure, since both contain high concentrations of heme-containing proteins, which can act as pro-oxidants (Halliwell and Gutteridge, 1999). Specifically, *G. dibranchiata* erythrocytes contain hemoglobin (Mangum et al., 1989), while liver cells have high concentrations of CYP450. Consistent with this, Eghbal et al. (2004) showed that addition of the CYP450 inhibitor benzylimidazole decreased sulfide cytotoxicity by up to 50% in rat hepatocytes. Antioxidant defenses of sulfide-adapted animals against sulfide exposure could include increased activities or concentrations of antioxidant enzymes such as superoxide dismutase and catalase, but also increased concentrations of low molecular mass free radical scavengers. Interestingly, glutathione and hypotaurine, which are present in high concentrations in tissues of sulfide-adapted invertebrates (Pruski et al., 1997; Yancey et al., 2002; Yin et al., 2000), are both potent free radical scavengers (Halliwell and Gutteridge, 1999).

Significance

Many sulfidic environments have communities of invertebrates adapted to sulfide concentrations from 0.05 mmol l^{-1} to more than 12 mmol l^{-1} (Childress and Fisher, 1992; Grieshaber and Völkel, 1998; Urcuyo et al., 2003; Van Dover and Lutz, 2004). Although intact sulfide detoxification mechanisms may reduce sulfide toxicity for the majority of cells in these animals, integumentary tissues, epithelial tissues at respiratory surfaces and circulating respiratory cells are still exposed to potentially toxic sulfide concentrations. If the irreversible mitochondrial toxicity observed in the present study occurs in epithelial cells of sulfide-adapted animals in their natural environment, then it may be an important factor affecting sulfide tolerance by necessitating substantially upregulated mitochondrial repair or degradation and biogenesis. In support of this, some histological studies of epithelial tissues from sulfide-adapted, sulfide-exposed invertebrates have identified mitochondrial swelling and the presence of electron-dense mitochondrial matrices and granules (Duffy and Tyler, 1984; Janssen and Oeschger, 1992; Jouin and Gaill, 1990; Menon and Arp, 1993, 1998; Menon et al., 2003), which may be evidence of mitochondrial injury, but such changes have not been seen in all studies (Dubilier et al., 1997). Irreversible mitochondrial depolarization from sulfide would be likely to result in MOMP, which would be followed by release of mitochondrial pro-apoptotic factors into the cytoplasm and cell death soon thereafter (Green and Kroemer, 2004). Indeed, the smooth appearance of *G. dibranchiata* erythrocytes exposed to sulfide (Fig. 1) suggests cellular swelling, which is a characteristic of cell death following PT pore opening in mammalian cells (Nieminen, 2003). Once mitochondrial injury and depolarization begin, the only mechanism available to the organism to prevent cell death would be autophagic ingestion of the injured mitochondria (Bauvy et al., 2001; Shintani and Klionsky, 2004). Indeed, it has been proposed that electron-dense organelles, which are characteristic of epidermal tissues in sulfide-adapted annelids (e.g. Giere et al., 1988; Hourdez and Jouin-Toulmond, 1998; Jouin and Gaill, 1990; Jouin-Toulmond et al., 1996; Menon et al., 2003), represent 'secondary lysosomes' containing autophagocytosed, sulfide-damaged and degenerating mitochondria (Arp et al., 1995). Whether mitochondrial injury, represented by depolarization, results in increased mitochondrial autophagy remains to be determined.

Conclusions

In this study, we demonstrate that mitochondria in *G. dibranchiata* erythrocytes can be successfully labeled with the $\Delta\Psi$m indicator dyes JC-1 and TMRM and that these dyes show changes in fluorescence emission characteristic of mitochondrial depolarization when the erythrocytes are exposed to the mitochondrial uncoupler CCCP. When erythrocytes were exposed to various concentrations of sulfide for 1 h, JC-1 and TMRM showed a similar, dose-dependent change in fluorescence, suggesting that sulfide caused loss of $\Delta\Psi$m. This change was irreversible over 5 h and was not seen to the same extent with azide or cyanide, which both inhibit COX, or with other mitochondrial electron transport chain inhibitors, with the possible exception of rotenone. JC-1 and TMRM in cell-free incubation buffer showed no change in fluorescence spectra when exposed to sulfide, which increases our confidence that the changes in fluorescence emission observed in these dyes when loaded into *G. dibranchiata* erythrocytes exposed to sulfide are not due to a chemical interaction between each dye and sulfide. Furthermore, erythrocytes that had been exposed to sulfide for 1 h, then rinsed in sulfide-free buffer, incubated in sulfide-free conditions for up to 5 h and only then loaded with TMRM, showed fluorescence similar to that of cells immediately following exposure to sulfide or CCCP (Fig. 5). Since it is highly improbable that any free sulfide would have remained after 5 h, it is likely the fluorescence change represents loss of $\Delta\Psi$m.

The mechanism by which sulfide irreversibly depolarizes mitochondria is not clear. Although the PT pore inhibitors CsA and TFP have been shown to improve cell survival during sulfide exposure, we found that they did not prevent loss of $\Delta\Psi$m. One potential mechanism of mitochondrial injury is free radical damage from increased ROS production. This was supported by the observation that H_2DCFDA oxidation and MitoSOX^{TM} oxidation were increased two- to threefold in

erythrocytes exposed to sulfide, suggesting increased oxidative stress and superoxide production, respectively. However, it remains to be determined whether ROS production is a contributing factor to, or a consequence of, $\Delta\Psi$m loss during sulfide exposure.

List of abbreviations

ANT	adenine nucleotide translocator
CCCP	carbonyl cyanide *m*-chlorophenylhydrazone
COX	cytochrome *c* oxidase
CsA	cyclosporine A
CyP-D	cyclophilin D
DCF	dichlorofluorescein
ETC	electron transport chain
H_2DCFDA	2′,7′-dichlorodihydrofluorescein diacetate
JC-1	5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide
MOMP	mitochondrial outer membrane permeabilization
PT	permeability transition
ROS	reactive oxygen species
TFP	trifluoperazine
TMRM	tetramethylrhodamine methyl ester
VDAC	voltage-dependent anion channel
$\Delta\Psi$m	mitochondrial electrical potential

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