The Journal of Experimental Biology 201, 1393–1403 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
JEB1352

NEUROMUSCULAR SENSITIVITY TO HYDROGEN SULFIDE IN THE MARINE INVERTEBRATE *URECHIS CAUPO*

DAVID JULIAN, WENDY E. DALIA AND ALISSA J. ARP*

Romberg Tiburon Center for Environmental Studies and Department of Biology, San Francisco State University, PO Box 855, Tiburon, CA 94920-0855, USA

*e-mail: aarp@sfsu. edu

Accepted 22 January; published on WWW 20 April 1998

Summary

Hydrogen sulfide (HS) is a well-known inhibitor of aerobic respiration *via* its reversible binding of mitochondrial cytochrome *c* oxidase, but recent studies have suggested that HS may have other non-respiratory actions. We have studied the effects of HS on spontaneous and evoked contractions *in vitro* under hypoxic and anoxic conditions in nerve–muscle preparations from the echiuran worm *Urechis caupo*. Contraction amplitude in response to electric field stimulation under anoxic conditions was completely abolished by HS within minutes in a classic dose–response relationship (K_d=31 mmol l^{-1}, r^2=0.86). Exposure of body wall and esophagus to HS *in vitro* for up to 6 h demonstrated that the contraction amplitude and frequency of spontaneous activity were relatively insensitive to anoxia, but that the sensitivity to HS was similar to that seen in field-stimulated muscle (K_d=2.7–32 mmol l^{-1}). The toxic effects of HS were reversible, with almost complete recovery under anoxic conditions within the first hour. These data indicate that HS at millimolar concentrations can directly inhibit muscle contraction. Although the mechanism of this action is unknown, it does not appear to involve metabolic pathways or oxygen transport.

Key words: muscle, contraction, toxicity, Echiura, *Urechis caupo*, hydrogen sulfide.

Introduction

Hydrogen sulfide (HS) is a metabolic poison that is typically fatal to animals relying solely on aerobic respiration. The best-characterized toxic effects of HS are its reversible inhibition of cytochrome *c* oxidase, the terminal element of the mitochondrial electron transport chain, and its reaction with hemoglobins to form sulfhemoglobin and sulfmyoglobin, thereby greatly decreasing their oxygen affinity. The action of HS on cytochrome *c* oxidase is more potent than that of cyanide, with 50 % inhibition at concentrations as low as 1 μmol l^{-1} (Nicholls, 1975; National Research Council, 1979). HS is a weak acid existing in three charge states, H_2S, HS^- and S^{2-}, that occurs naturally in a number of marine and aquatic environments, including hydrothermal vents, cold seeps, mudflats and marshes. In each of these environments, diverse communities of animals have been found that are specifically adapted to the presence of HS (Somero *et al.* 1989; Childress, 1995; Fisher, 1995).

Animals adapted to sulfidic environments have typically been classified into four categories: (1) animals that tolerate the toxicity of HS by remaining in anaerobiosis throughout an exposure (Oeschger and Storey, 1990), (2) animals that remain aerobic during exposure by excluding HS or oxidizing it to less toxic forms, but that have no metabolic HS requirement (Vetter *et al.* 1987; Vismann, 1991; Arp *et al.* 1995), (3) animals that harbor symbiotic HS-oxidizing bacteria, but that remain aerobic themselves either by oxidizing HS or by sequestering HS from aerobic tissues (Powell and Somero, 1986; Somero *et al.* 1989), and (4) animals that may directly exploit HS as an energy source *via* mitochondrial HS oxidation (Powell and Somero, 1986; Lee *et al.* 1996; see Völkel and Grieshaber, 1996).

Implicit in these descriptions is the assumption that the primary and most important toxicity of HS is its inhibitory effect on aerobic metabolism. However, many studies, primarily of vertebrates and especially of mammals, suggest that HS has other, non-respiratory toxic effects (for a review, see Reiffenstein *et al.* 1992). A number of reports have demonstrated an effect of HS on vertebrate nerve and muscle tissue, with reported actions including inhibition of axonal conduction (Beck *et al.* 1983), inhibition of synaptic transmission (Abe and Kimura, 1996), interaction with nitric oxide signaling (Kruszyna *et al.* 1985; Hosoki *et al.* 1997), alteration of ion channel activity (Kombian *et al.* 1993), effects on RNA synthesis (Beauchamp *et al.* 1984) and, during development, changes in neurotransmitter content and neuronal growth (Roth *et al.* 1995; Hannah and Roth, 1991). However, in most of these studies, the actions of HS were not dissociated from the effects of HS on metabolism. Thus, it is

unclear whether HS might have acted directly on, for example, nerve cell membranes, receptors, ion channels or intracellular signaling mechanisms.

Sedentary invertebrates are abundant in many sulfidic environments, and in some cases comprise the majority of the macrofauna. The behavior of such animals, which includes bivalves, tube worms and some burrow-dwelling animals, consists primarily of rhythmic muscular movements for feeding and gas exchange. In many cases, it is possible to isolate nerve and muscle tissues from these animals such that these rhythmic patterns are retained *in vitro*. To begin investigating whether non-respiratory toxicity of HS is an important factor for HS-adapted marine invertebrates, we studied the effects of HS on both rhythmic and evoked muscle contractions using *in vitro* nerve–muscle preparations from the marine echiuran worm *Urechis caupo*.

U. caupo lives in U-shaped burrows in mud- and sandflats along the California coast (Fisher and MacGinitie, 1928; Arp *et al.* 1992). *U. caupo* continuously circulates water through its burrow with anterior-to-posterior peristaltic waves along its muscular body wall, supplying oxygen, bringing food particles into the mucus net and expelling wastes and gametes from the burrow. While many *U. caupo* live subtidally, a subpopulation lives in the intertidal zone where the HS concentration reaches 25–30 μmol l^{-1} and oxygen tension drops to as low as 30 % of air saturation during low tides (Arp *et al.* 1992). In comparison with HS concentrations found in other sulfidic habitats, which can extend well into the millimolar range, the concentrations experienced by *U. caupo* are rather low. Nonetheless, *U. caupo* possesses a variety of mechanisms to detoxify or sequester HS, including heme-based oxidation of HS to thiosulfate and HS-binding by heme compounds (for a review, see Arp *et al.* 1995).

U. caupo has a very simplified anatomical arrangement consisting of a thick, muscular body wall surrounding an undivided coelomic cavity which contains paired sets of gonads, a pair of excretory organs termed anal vesicles, a ventral nerve cord suspended within the coelomic cavity by several hundred pairs of peripheral nerves, and a long intestine that folds back on itself several times, terminating in the hindgut and cloaca. The hindgut, which is suspended within the coelomic cavity and connected to the body wall by strands of muscle tissue, is tidally ventilated with water, and approximately half of the total oxygen uptake of *U. caupo* at normoxia occurs across this tissue surface (Julian *et al.* 1996). *U. caupo* has no vascular system, and the coelomic fluid, which contains hemoglobin in large coelomocytes, circulates freely within the coelomic cavity, bathing all of the organs, including the entire serosal surfaces of the body wall and hindgut.

There are specific advantages in using *U. caupo* for studies of neuromuscular activity *in vitro*. Unlike invertebrates from the sea floor, *U. caupo* is readily obtained from the local environment during low tides and is easily maintained in the laboratory in standard seawater aquaria. Additionally, the very simple anatomy of *U. caupo* and its lack of a closed vascular system permit nearly normal perfusion of isolated tissue preparations *in vitro*. Finally, the most obvious two muscular activities of *U. caupo*, the constant pumping of water through its burrow and the periodic ventilation of its hindgut for gas exchange, are maintained in isolated nerve–muscle preparations for many hours (Lawry, 1966).

Materials and methods

Animal collection and handling

Urechis caupo were collected during low and moderate tides from two California locations: Elkhorn Slough, Monterey County, and Pillar Point Harbor (Princeton Harbor), San Mateo County. Worms were collected with a polyvinylchloride suction gun or were hand-dug from burrows. They were then transported to the laboratory within 2–3 h of collection, where they were maintained unfed in recirculating seawater aquaria for up to 3 weeks. Experimental animals weighed between 40 and 80 g wet mass.

Reagents and solutions

Bathing solution was made from artificial sea water (Instant Ocean, Aquarium Systems) with 0.1 % glucose and 0.05 mol l^{-1} Tris buffer (Sigma). HS was added, as needed, from stock solutions of 100 mmol l^{-1} or 500 mmol l^{-1} $Na_2S{\cdot}9H_2O$ or from a saturated Na_2S stock solution maintained at 4 °C. Osmolality was adjusted to 1000 mosmol kg^{-1}, as measured with a vapor pressure osmometer (Wescor). Anoxic solutions were prepared by deoxygenation by vigorously bubbling pure N_2 gas until the oxygen tension was less than 0.5 kPa (measured using a Clark-style oxygen electrode calibrated against fresh 2 % sodium sulfite). Bathing solutions were adjusted to pH 7.4 by titration with NaOH or HCl. All experiments were conducted at room temperature (20–22 °C).

Tissue preparation

Body wall nerve–muscle preparations were dissected as muscle tissue sheets approximately 1 cm wide and 2 cm long, with associated nerve tissue, including the attached nerve cord. These preparations were obtained from three positions in the worm: the anterior section (including the proboscis), the middle section and the posterior section (including a portion of the hindgut). In addition to body wall nerve–muscle preparations, some experiments also utilized preparations of esophagus, which also contracts spontaneously *in vitro*. Esophageal preparations were dissected from the muscular anterior-most portion of the intestine. Body wall and esophageal preparations were stored in aerated bathing solution until needed (up to 3 h).

Measurement of spontaneous contractile activity

Body wall and esophageal nerve–muscle preparations were placed into a muscle chamber containing aerated bathing solution. The tissue was suspended between an isometric force transducer (FORT250g for body wall, FORT10g for esophagus, World Precision Instruments) and the base of the chamber with silk or cotton thread. The force transducer was

mounted above the chamber with a manipulator, which allowed adjustment of preload tension. The force transducer output was low-pass-filtered at 100 Hz, amplified (CP202 preamplifier, Sable Systems) and digitally recorded at 2–10 samples s^{-1} (Sable Systems PC-based analog to digital converter).

A study of spontaneous activity at various P_{O_2} levels was performed on body wall preparations. In this experiment, the baseline activity of the preparation was recorded after an initial 10 min equilibration period. The P_{O_2} of the bathing solution was then altered by bubbling it with mixtures of N_2 and O_2 (mixed from compressed gas with a GF-4 gas-mixing flowmeter, Cameron Instrument Company) to normoxia (P_{O_2}=21 kPa), hypoxia (P_{O_2}=4–5 kPa), anoxia (P_{O_2}<0.5 kPa) or hyperoxia (P_{O_2}>40 kPa). The order of P_{O_2} treatments for each tissue preparation was randomized. The oxygen tension of the bathing solution was continuously monitored using a Clark-style oxygen electrode. The bathing solution was continuously stirred on a magnetic stir plate to ensure thorough mixing. Initial control experiments were conducted for 2 h at each experimental oxygen tension to ensure that any change in contraction pattern was a factor of bathing solution P_{O_2} and not of time.

To determine the concentration of HS required to inhibit muscle activity, body wall and esophagus nerve–muscle preparations were mounted in a recording chamber, as described above, and allowed to equilibrate for 1 h in anoxic bathing solution. At the end of the equilibration, a baseline recording of spontaneous activity was obtained. HS stock solution was then incrementally added to the bathing solution at 30 min intervals (together with water to maintain osmolality at 1000 mosmol kg^{-1}, and 0.5 mol l^{-1} HCl, if necessary, to maintain pH at 7.4). A recording of spontaneous activity was obtained just prior to each addition. For each tissue preparation, these additions were repeated until no spontaneous activity was evident.

Throughout the experiment, the bathing solution was kept well-stirred with a magnetic stirrer, and the pH was continuously monitored and adjusted. The gas space above the bathing solution was continuously flushed with N_2, but the solution itself was not bubbled with N_2 during the experiments, since this would tend to strip the volatile H_2S species of HS from the solution. For exposures to a single HS concentration lasting more than 30 min, the bathing solution was replaced every 60–90 min to ensure that the HS concentration was constant.

Direct nerve stimulation

For studies of evoked activity, muscle tissue from the mid section of the body wall was dissected out and mounted as described above, except that the anterior portion of the nerve cord leading to the muscle was left intact. The section of the nerve dissected free in these preparations was typically at least 2 cm in length. The anterior end of this nerve was inserted into a special stimulating electrode probe (Burn and Rand, 1960) that allowed the portion of the nerve between the stimulating wires to be continuously bathed in flow-through, aerated, HS-free bathing solution. The remainder of the nerve was exposed to the same solution as the muscle sheet. The nerve was periodically advanced through the stimulator to a 'fresh' segment to minimize any cumulative damaging effects of electrical stimulation. Preload on the muscle sheets was maintained at a constant tension (approximately 30 g) to compensate for any tissue stretch between stimulation experiments.

The nerve–muscle preparation was maintained in aerated bathing solution for 60 min while the spontaneous activity was continuously monitored. At 30 min and at 1 h, the nerve was stimulated with 20 ms 4–10 V biphasic pulses at a frequency of 100 Hz (Grass SD9 stimulator) until the evoked contraction appeared to be tetanized. The contraction was digitally recorded at 10 samples s^{-1} and analyzed off-line for peak contraction amplitude, time to peak amplitude (time lag between initiation of the stimulus and the peak amplitude for that contraction) and time to half-relaxation (the time required after the cessation of stimulation for the muscle tension to reduce by half). The bathing solution was then replaced with anoxic bathing solution, and monitoring of spontaneous contractile activity was continued for another hour. The nerve was then stimulated and the muscle response recorded after 30 and 60 min as before. HS was then added from a stock solution (pH 7.4) to bring the total HS concentration to between 1 and 5 mmol l^{-1}. Monitoring and recording of spontaneous activity and stimulation of evoked activity were then continued for an additional 60 min as described above.

At the end of the HS exposure, a section of the nerve between the stimulator probe and the muscle was gently crushed using forceps, and the nerve was again stimulated as before. The inability of this stimulus to evoke any contraction after the crush confirmed that the previous responses were not due to direct electrical stimulation of the muscle.

Electric field stimulation

For field stimulation, body wall muscle strips, 1–2 cm long and 2–3 mm wide, were dissected out and placed in cold bathing solution until needed. Suitable strips were mounted in an acrylic muscle chamber (inside chamber dimensions 4 cm long, 1 cm wide, 1 cm deep) with a panel of platinum mesh attached lengthwise on either side of the muscle strip. Using cotton thread, one end of the muscle strip was then secured to one end of the chamber, and the other end was passed under a glass rod at the other end and up to a force transducer (FORT10g, World Precision Instruments) mounted on a micromanipulator above the chamber. Anoxic bathing solution was continuously circulated through the chamber from a reservoir by a peristaltic pump at 3 ml min^{-1}. This perfusion continued throughout the remainder of the experiment. After a strip had been mounted in the chamber, current pulses were passed between the platinum mesh electrodes (and thereby across the muscle strip) every 5 min from an electronic stimulator (Grass S88) for the duration of the experiment. These pulses were 15 Hz, 20 ms duration biphasic pulses. The electrical potential across the chamber was

100 V, with a total current of 75 mA. The stimulation was continued for each event until the contraction amplitude reached a plateau.

During an initial 30 min equilibration period for each new tissue strip, optimal preload was determined by gradually increasing the tension on the tissue, using the micromanipulator, until the contraction was maximal. This tension was then maintained throughout the remainder of the experiment. After a baseline recording had been established, the HS concentration of the bathing solution was increased incrementally at 20 min intervals until no contraction could be evoked. Data presented for each HS concentration are from the last stimulus of each 20 min exposure period. All amplitudes are reported relative to the amplitude for that tissue at the end of the anoxia control period.

Statistical analyses and data presentation

Values are presented as means ± one standard deviation (S.D.). Single-factor analysis of variance (ANOVA) or within-subjects ANOVA, as indicated, followed by Tukey or Student–Newman–Keuls multiple-comparison tests was used to test for significant differences between measurements (Zar, 1984; Glantz, 1997). Differences were considered significant at $P<0.05$. Amplitude and frequency results from spontaneous muscular activity data were analyzed using specially written software, with the intent of reducing investigator bias. Dose–response data were fitted to the equation $y=K_d/([HS]+K_d)$, where y is the tissue response relative to the anoxic control, [HS] is the HS concentration in mmol l^{-1}, and K_d represents the HS concentration at which the tissue response is half of the control value (Hille, 1992). Non-linear curve fitting was performed using commercial software (DeltaGraph v. 4.02, SPSS Inc.).

Results

Response of spontaneous body wall activity to varying P_{O_2} *and micromolar HS concentrations*

Nerve–muscle preparations contracted rhythmically and spontaneously *in vitro*. At normoxia, preparations isolated from both the anterior and posterior regions of the worm demonstrated a rhythmic pattern with a frequency of approximately 5.8 contractions min^{-1} (Fig. 1). Exposure to anoxia initiated a superimposed lower-frequency pattern at approximately 0.6 contractions min^{-1}, which then remained throughout the duration of the experiment. For further analyses of rhythmic behavior, we considered only the underlying higher-frequency component.

The average amplitude of spontaneous contractions was not significantly affected by any of the P_{O_2} values tested (Fig. 2A,B, open bars). To normalize for differences between muscle mass and strength between tissue preparations, contraction amplitudes at each P_{O_2} in the figure are normalized to the average amplitude at normoxia. Anterior and posterior preparations from the body wall demonstrated a significant effect of P_{O_2} on the frequency of spontaneous activity only between normoxia and hypoxia (Fig. 2C,D, open bars). However, the frequency of spontaneous activity in these preparations was not significantly different from that at normoxia at either anoxia or hyperoxia (N=6). Midsection preparations were not affected by changes in P_{O_2} (N=6, data not shown).

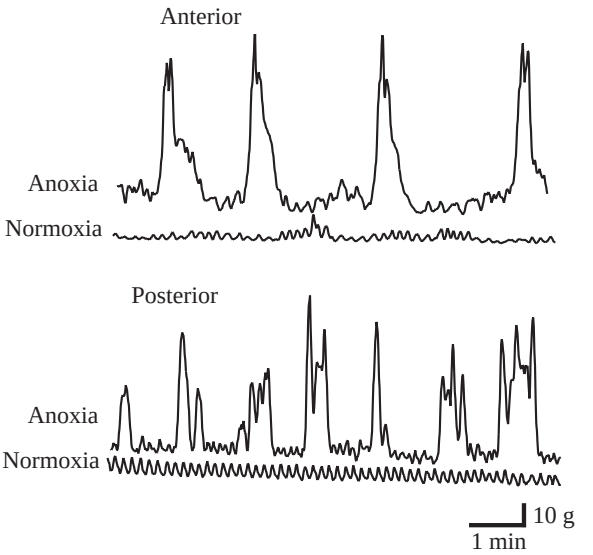

Fig. 1. Representative recordings of spontaneous activity in *Urechis caupo* body wall nerve–muscle preparations. Body wall nerve–muscle preparations isolated from the anterior (top tracings) or posterior (bottom tracings) of the worm were placed into a muscle chamber containing aerated (normoxic) or nitrogen-gassed (anoxic) bathing solution (artificial sea water with 0.1 % glucose and 0.05 mol l^{-1} Tris buffer). Spontaneous activity was recorded with an isometric force transducer. Scale bars represent time (horizontal bar, in min) and tension (vertical bar, in g).

Six anterior and posterior body wall preparations were exposed to micromolar HS concentrations (80–300 μmol l^{-1}) for 20 min under either normoxic or anoxic conditions (three tissues tested at each condition). Because the addition of HS consistently caused a brief (<2 min) increase in baseline muscle tension, amplitude data presented below were collected after this contraction had subsided. Regardless of whether oxygen was present in the bathing solution, the average peak amplitude and frequency of spontaneous activity were not significantly affected by HS exposure (Fig. 2A–D, filled bars). As described above, amplitude data were normalized to the average amplitude in normoxic, HS-free conditions.

Response of direct nerve stimulation to anoxia and millimolar HS concentrations

To determine whether the ability of the nerve cord either to conduct impulses or to initiate depolarization of the muscle could be inhibited by millimolar HS concentrations, we analyzed the contractions resulting from direct electrical stimulation of the nerve cord in four body wall nerve–muscle preparations following a 1 h exposure to 1–5 mmol l^{-1} HS under normoxic conditions. To confirm that any effects of HS were not due to inhibition of aerobic metabolism, each tissue sample was exposed first to 1 h of normoxia, followed by 1 h

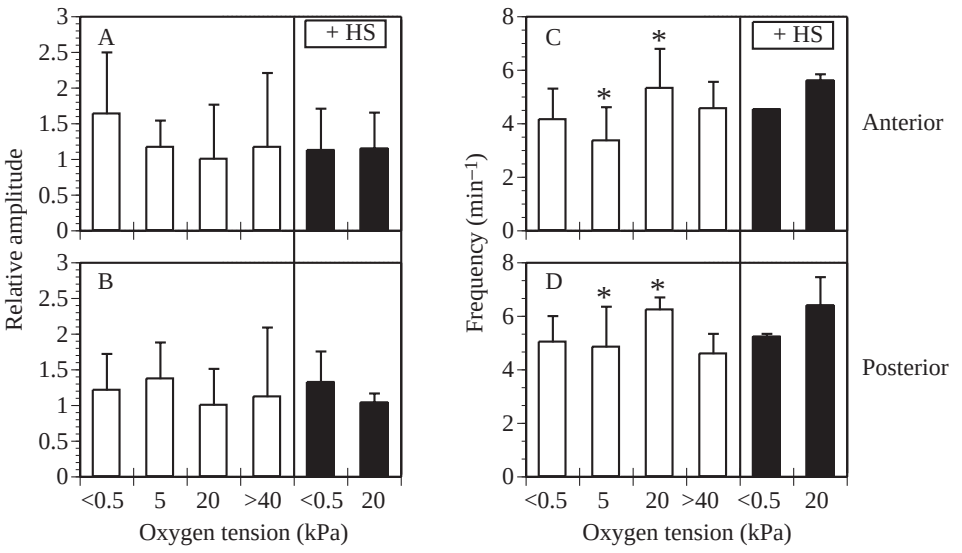

Fig. 2. Response of *Urechis caupo* body wall nerve–muscle preparations to varying levels of oxygen with and without added HS. (A,B) Relative amplitude. All amplitude values were normalized to the average amplitude under normoxic, HS-free conditions. (C,D) Frequency. These values were not normalized. Body wall nerve–muscle preparations isolated from the anterior (A,C) or posterior (B,D) of the worm were placed into a muscle chamber containing bathing solution equilibrated to oxygen tensions of <0.5 kPa (anoxic), 5 kPa (hypoxic), 20 kPa (normoxic) or >40 kPa (hyperoxic). HS was added to a concentration of 80–300 μmol l^{-1} with an oxygen tension of either less than 0.5 or 20 kPa (filled bars). Spontaneous activity was recorded with an isometric force transducer. Values are means + S.D. (N=6). Significantly different values within a plot are indicated by asterisks.

of anoxia, and finally 1 h of anoxia with 1–5 mmol l^{-1} HS. The nerve was stimulated at the beginning, at the midpoint and at the end of each exposure. Functional measurements of muscle activity, such as the time required to reach peak force, the maximum force achieved during stimulation and the time required to reach half-relaxation after cessation of the stimulus were not significantly affected by changes in P_{O_2} or by the addition of 1–5 mmol l^{-1} HS during anoxia (Fig. 3).

Response of spontaneous activity to HS

To determine whether *U. caupo* tissues were completely insensitive to HS, body wall and esophagus nerve–muscle preparations were exposed to stepwise increases of HS up to a maximum concentration of 140 mmol l^{-1} under anoxic conditions. The exposure duration at each concentration was at least 20 min, and each tissue was tested until a concentration was reached that inhibited the contraction amplitude by at least 90 %. Each tissue was typically exposed to at least four HS concentrations. Nine body wall and four esophagus preparations were tested, with a minimum of three preparations at each concentration, except as noted. To confirm that any effects were not due to inhibition of aerobic metabolism, the response to each HS concentration was normalized to the response recorded after a 1 h exposure to anoxia in the absence of HS, performed at the very beginning of each experiment.

Body wall nerve–muscle preparations exhibited practically normal spontaneous activity up to 10 mmol l^{-1} HS (Fig. 4). Above this concentration, the average amplitude and frequency of spontaneous activity decreased and became consistently absent at 100 mmol l^{-1} HS. Fitting each body wall data set to the dose–response curve yielded a frequency K_d of 32 mmol l^{-1} (r^2=0.49) and an amplitude K_d of 15 mmol l^{-1} (r^2=0.76).

Esophagus preparations contracted regularly, as did the body wall preparations (data not shown). The effect of HS on the amplitude of spontaneous esophagus activity was similar to that on body wall. Amplitude was almost normal at 1 mmol l^{-1} HS, but was virtually absent at 32 mmol l^{-1} HS (Fig. 5). Fitting a dose–response curve yielded a K_d of 2.7 mmol l^{-1} (r^2=0.40), indicating a somewhat higher sensitivity than that of the body wall. However, the frequency of spontaneous esophagus activity was not affected by HS (P=0.14) and did not fit the dose–response curve equation.

Response of body wall activity to long-term exposure at millimolar HS concentrations

Although the exposures in the previous dose–response experiments were of relatively short duration, each tissue sample was exposed to gradually increasing HS concentrations over a period of 2–3 h. Thus, it is possible that the dose–response effects were due not to the higher concentration, but rather to the longer duration of the exposure. To confirm that this was not the case, body wall nerve–muscle preparations were exposed to anoxia with either 5 mmol l^{-1} HS for 2.5 h or 10 mmol l^{-1} HS for 6 h, with 10 tissue preparations tested at each concentration (Fig. 6). At both concentrations, the preparations continued spontaneous activity throughout the duration of the exposure. Contraction frequency was unchanged, on average, at both HS concentrations. Contraction amplitude was unaffected by a 2.5 h exposure to 5 mmol l^{-1} HS, but was significantly (P<0.05 by ANOVA) and consistently decreased after a 3 h exposure to 10 mmol l^{-1} HS. However, contraction activity persisted throughout the exposure, remaining at 20 % of the anoxic control level after 6 h.

Reversibility of HS toxicity in body wall

To determine whether the inhibitory effects of HS on spontaneous activity are reversible, four body wall nerve–muscle preparations were exposed to 10 mmol l^{-1} HS

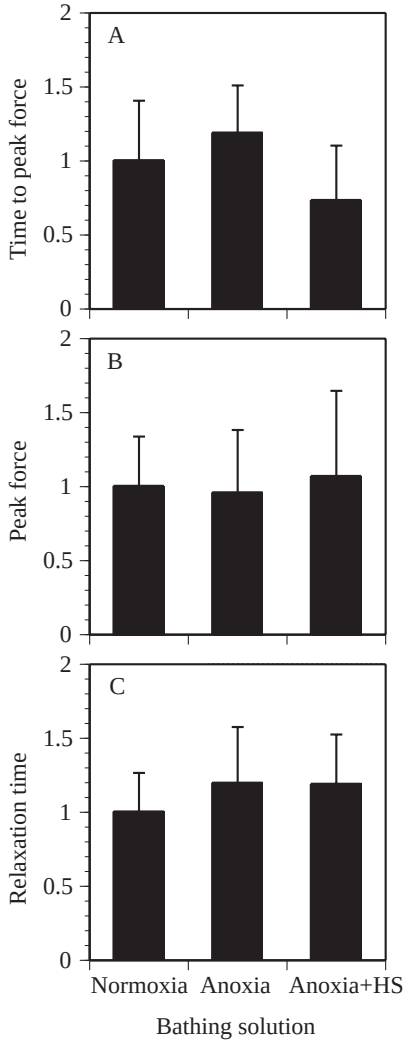

Fig. 3. Response of *Urechis caupo* body wall nerve–muscle preparations to direct nerve stimulation. All data are presented relative to the response at normoxia. (A) Time required for contraction to reach peak force; (B) peak force; (C) time required for relaxation to reach half of the peak force. Muscle tissue from the mid section of the body wall was dissected out and mounted in a nerve chamber such that the anterior portion of the nerve cord leading to the muscle was left intact and was inserted into a stimulating electrode probe. The remainder of the nerve was exposed to the same solution as the muscle sheet. The nerve was stimulated twice per hour with tetanizing pulses. Contraction force was measured with an isometric force transducer. Tissues were exposed to bathing solution equilibrated with air (normoxia) or nitrogen (anoxia). HS was added to anoxic bathing solution to a concentration of 1–5 mmol l^{-1} (Anoxia + HS). Values are means + S.D. (N=4).

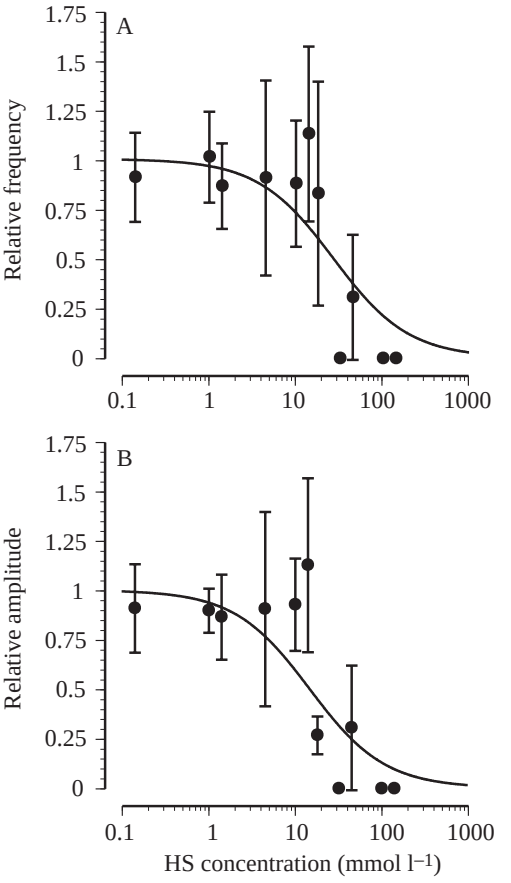

Fig. 4. Dose–response characteristics of spontaneous activity in *Urechis caupo* body wall nerve–muscle preparation to HS. (A) Effect of HS on the relative frequency of spontaneous muscular activity. (B) Effect of HS on the relative amplitude of spontaneous activity. Values are means ± S.D. (N=9 preparations, with 3–4 preparations tested at each concentration) and are presented relative to the value after a 1 h exposure to anoxia in the absence of HS. Details of the dose–response curve are given in Materials and methods. Frequency K_d=32 mmol l^{-1} (r^2=0.49, P<0.005), amplitude K_d=15 mmol l^{-1} (r^2=0.76, P<0.005).

during anoxia for 90 min, followed by replacement of the bathing solution with anoxic, HS-free bathing solution. The recovery of spontaneous activity was then followed for 60–100 min. As expected, 10 mmol l^{-1} HS reduced the average contraction amplitude to approximately 20 % of the anoxic control and had a smaller effect on the contraction frequency. In every case, however, removal of the HS resulted in a gradual recovery of the relative amplitude towards the control value (Fig. 7). Interestingly, the removal of HS resulted in a consistently increased contraction frequency, averaging about twice the anoxic control frequency after 60 min. It is important to note that the HS 'recovery' period was under anoxic conditions, so reversibility is likely to be due solely to reversal of binding and/or HS diffusion out of the tissues, as opposed to HS oxidation.

Response of field-stimulated muscle activity

Body wall muscle strips were depolarized by electric field stimulation to bypass the nerve–muscle conduction pathway. This allowed the effects of HS on muscle to be isolated from those on nerves or on the nerve–muscle synapse. Muscle strips were exposed to HS concentrations ranging from 220 μmol l^{-1} to 140 mmol l^{-1} under anoxic conditions (N=6 strips, with 3–4

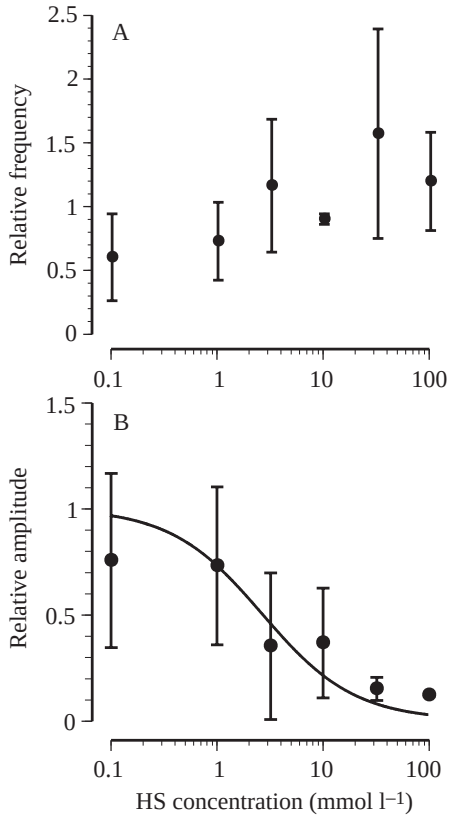

Fig. 5. Dose–response characteristics of spontaneous activity in *Urechis caupo* esophagus preparations to HS. (A) Effect of HS on the relative frequency of spontaneous muscular activity. (B) Effect of HS on the relative amplitude of spontaneous activity. Values are means ± S.D. (N=4 tissue preparations at each concentration, except for 10, 32 and 100 mmol l^{-1}, for which the data represent the average of three, two and two preparations, respectively) and are presented relative to the value after a 1 h exposure to anoxia in the absence of HS. Details of the dose–response curve are given in Materials and methods. Amplitude K_d=2.7 mmol l^{-1} (r^2=0.40, P<0.005). Frequency data did not fit the dose–response curve.

strips tested at each concentration, a total of 34 trials). Field stimulation produced a clear dose–response relationship between HS concentration and contraction amplitude (Fig. 8) with a K_d of 31 mmol l^{-1} (r^2=0.86).

Discussion

The control of rhythmic behavior in Urechis caupo

In addition to the finding of HS actions in muscle, the present report also provides new information about the activity and location of pacemaker sites in *U. caupo*. Evidence of ganglionic processes in echiurans has never been reported (Pilger, 1993), but Lawry (1966) suggested that two distinct pacemakers located in the anterior and posterior regions of the body wall or intestine control peristalsis of both the body wall and the hindgut, initiating ventilatory, feeding and burrowing activity. In intact animals, body wall peristalsis occurs at an average frequency of 12 contractions min^{-1} at normoxia (Julian *et al.* 1996), which is twice the frequency of spontaneous contractions seen in the body wall nerve–muscle preparations *in vitro* (5.8 contractions min^{-1}). The lower-frequency pattern, initiated by hypoxia *in vitro*, occurred at approximately 0.6 contractions min^{-1}, which is very similar to the 0.5 contractions min^{-1} frequency of hindgut ventilation contractions seen in whole animals (Julian *et al.* 1996). Since both the high- and low-frequency patterns were seen in nerve–muscle preparations isolated from either the anterior or posterior portions of the worm, the pacemaker sites for both burrow irrigation and hindgut ventilation appear to be distributed throughout the animal.

In intact animals, body wall peristaltic frequency, but not hindgut ventilation frequency, is sensitive to changes in ambient P_{O_2} (Julian *et al.* 1996). We did not see this sensitivity in the preparations *in vitro*. Two explanations for this difference are that responsiveness of peristaltic frequency to P_{O_2} may require an intact nervous system or that peristaltic frequency is normally modulated by signals from the coelomic fluid, such as metabolites or hormones. In contrast to our findings, Lawry (1966) reported that body wall nerve–muscle preparations did, in fact, respond to changes in bathing solution P_{O_2} with an alteration in contraction frequency. Unfortunately, this information was presented without statistics or a description of variability, so it is difficult to make quantitative comparisons.

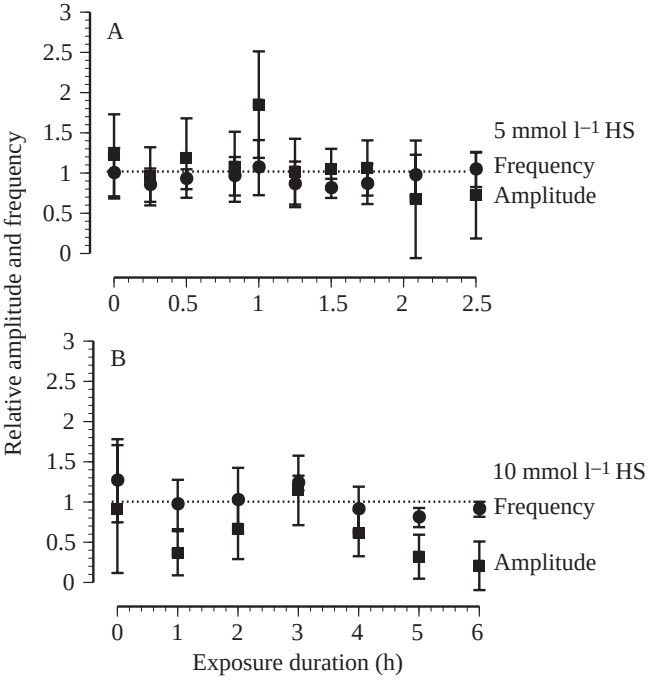

Fig. 6. Effects of long-term exposure to 5 and 10 mmol l^{-1} HS on *Urechis caupo* body wall nerve–muscle preparations. Tissues were exposed to anoxia with either 5 mmol l^{-1} HS for 2.5 h (A) or 10 mmol l^{-1} HS for 6 h (B). Values are means ± S.D. (N=10 tissue preparations for each panel, with each data point representing 2–4 preparations) and are presented relative to the value after a 1 h exposure to anoxia in the absence of HS. Circles, frequency; squares, amplitude.

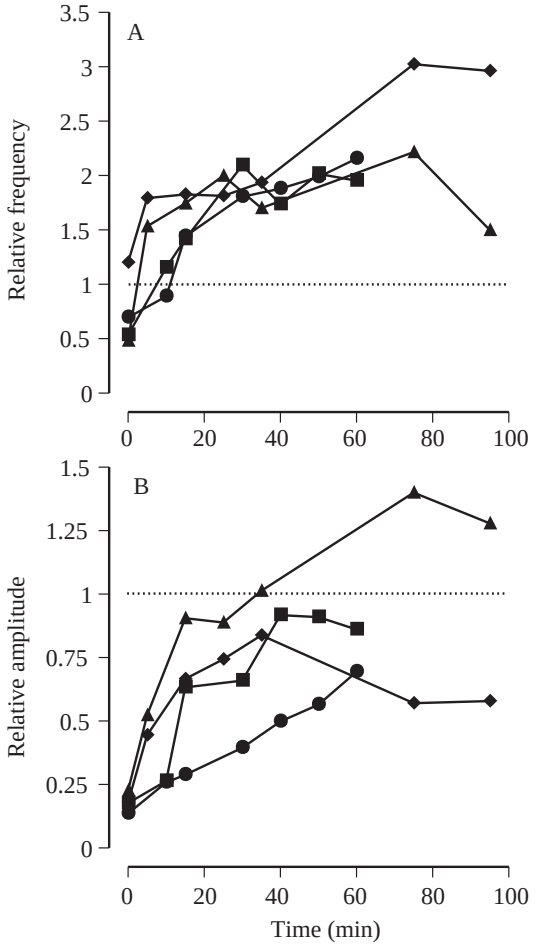

Fig. 7. Recovery of spontaneous activity in *Urechis caupo* body wall nerve–muscle preparations from a 90 min exposure to 10 mmol l^{-1} HS. Time zero indicates the beginning of HS washout in anoxic bathing solution. (A) Recovery of contraction frequency from HS inhibition. (B) Recovery of contraction amplitude from HS inhibition. The data from four preparations are presented. Values are relative to the control responses in anoxic, HS-free bathing solution.

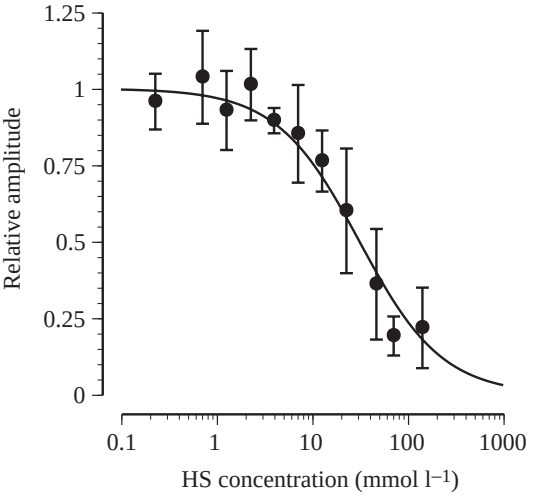

Fig. 8. Dose–response characteristics of *Urechis caupo* muscle strips stimulated with tetanizing, 100 V electric field pulses. The HS concentration of the anoxic bathing solution was increased incrementally at 20 min intervals until no contraction could be evoked. Tissues were stimulated every 5 min throughout the experiment. Data presented for each HS concentration are from the last stimulus of each 20 min exposure period. All amplitudes are reported relative to the amplitude for that tissue at the end of an initial control period in anoxic, HS-free bathing solution. Values are means ± S.D. (N=6 strips, with 3–4 strips tested at each concentration, 34 trials total). Details of the dose–response curve are given in Materials and methods. Amplitude K_d=31 mmol l^{-1} (r^2=0.86, P<0.001).

Non-cytochrome actions of HS on nerve and muscle

Little information is available regarding the effects of HS on muscle activity. In this study, we have demonstrated the first evidence of a direct action of HS on spontaneous muscle contraction. Bhambhani *et al.* (1997) examined skeletal muscle biopsies from exercising humans exposed to H_2S gas, but their analyses involved only assays for metabolic markers of anaerobic and aerobic metabolism. To our knowledge, only two previous studies have examined the effects of HS on muscle contraction. In both studies, HS, in combination with various NO-releasing compounds, was applied to mammalian smooth muscle that had already been placed in a state of contraction by a neurotransmitter, such as acetylcholine or norepinephrine. It was found that HS reversed the action of some NO-releasing compounds (Kruszyna *et al.* 1985), but augmented the action of others (Kruszyna *et al.* 1985; Hosoki *et al.* 1997). Hosoki *et al.* (1997) also demonstrated that, in guinea pig ileum and portal vein but not in thoracic aorta, HS alone at concentrations of 0.3–1 mmol l^{-1} could almost completely reverse the response to exogenous acetylcholine and norepinephrine.

In a whole-animal study, Vetter *et al.* (1987) tested the effects of HS on heart rate and scaphognathite beat frequency in several decapod crustaceans bathed in aerated sea water. HS up to 1.4 mmol l^{-1} (the highest concentration tested) increased scaphognathite beat frequency and had no effect on heart rate in a hydrothermal vent crab. However, as a result of the high HS-oxidizing capacity of this crab, the blood HS concentration never exceeded 100 μmol l^{-1}. Non-HS-adapted crustaceans showed less resistance to HS, with a 40–90 % decrease in heart rate and scaphognathite frequency resulting from exposure to 100–300 μmol l^{-1} HS.

In contrast to the limited research on muscle, a number of researchers have examined the effects of HS on vertebrate nervous tissue and have suggested that HS has direct effects on nerve activity. Beck *et al.* (1982, 1983) reported that short-term exposure to very high concentrations of HS (100 mmol l^{-1}) or H_2S gas (42 %) caused sciatic nerve bundles of *Rana pipiens* to become unresponsive or to have a weakened compound action potential. The actions of HS were found to be qualitatively different from those of cyanide. Beck *et al.* (1982, 1983) concluded that the inhibitory effect of HS on nerve function was independent of any actions on metabolism, and proposed that

an unknown direct site of HS toxicity exists on the nerve membrane. A similar conclusion was reached by Greer *et al.* (1995), who examined the effects of HS on the respiratory rhythm-generating pattern of rat brain *in vitro*. In this tissue, HS caused a reversible, dose-dependent decrease of up to 40 % in the frequency of rhythmic respiratory bursts within minutes of application. Although the ED_{50} was 120 μmol l^{-1}, the rhythmic pattern continued even in 600 μmol l^{-1} HS, the highest concentration tested. As in the studies described above, Greer *et al.* (1995) argued that, contrary to expectations, neuronal oxidative metabolism was not radically perturbed by HS, and proposed that HS can directly interfere with nerve membrane potential or transmitter function.

Relationship between anoxia tolerance and HS tolerance

If the sole effect of HS toxicity were inhibition of aerobic metabolism, then it might be assumed that animals with a high anaerobic capacity could readily tolerate a limited-duration HS exposure simply by entering anaerobiosis. In this case, the presence in *U. caupo* of well-developed HS detoxification mechanisms (Arp *et al.* 1995) might be seen as somewhat surprising since (1) *U. caupo* has an anaerobic tolerance of over 6 days, and (2) HS exposure in *U. caupo* is limited in concentration (maximum measured HS concentration of 70 μmol l^{-1}), duration (continuous exposure of at most 2 h) and frequency (few *U. caupo* burrows are exposed by low tides more than a few times per week; Arp *et al.* 1992).

Thus, the presence of elaborate detoxification mechanisms in *U. caupo* suggests that the function of detoxification is not only the maintenance of aerobic metabolism during tidal HS exposure, which might provide an energetic advantage even with the added metabolic cost of maintaining a detoxification system, but may also include the protection of the tissues from some, as yet undetermined, non-respiratory toxicity. Indeed, although anoxia tolerance and HS tolerance are strongly correlated across a range of invertebrates, when compared with anoxia alone, the addition of HS to anoxic water uniformly decreases survival time by 10–60 % (Theede *et al.* 1969; Groenendaal, 1980; Levitt and Arp, 1991). This finding suggests that HS has lethal effects beyond those of simply inhibiting aerobic metabolism.

Relevance of the results

Even though *U. caupo* is, in its natural environment, only known to experience HS concentrations of less than 100 μmol l^{-1}, the following two hypotheses illustrate how our finding of a toxic effect of HS with a K_d in the millimolar range may be of physiological relevance. First, the toxic effect of HS on muscle contraction may result from molecular interactions between HS and targets that are common to other tissue types, and our ability initially to recognize this interaction only in muscle may be its easily observable result, the inhibition of contraction. Indeed, although similar actions of HS in other tissue types may have a lower K_d, the effects may be more difficult to recognize. Second, although *U. caupo* does not normally experience millimolar concentrations of HS, many organisms do. If the muscle tissues of these other animals have the same HS sensitivity as those of *U. caupo*, then HS toxicity may be a significant factor in the distribution and physiology of these animals.

Does non-respiratory HS toxicity limit the distribution of HS-adapted animals?

Soft marine sediments are generally anoxic or severely hypoxic just a few centimeters below the surface. In these sediments, the HS concentration commonly approaches 9 mmol l^{-1} and can exceed 20 mmol l^{-1} (for a review, see Vismann, 1991). The complex ecological structure of this sulfidic environment, with its world-wide distribution, was first described almost 30 years ago (Fenchel and Riedl, 1970). Animals inhabiting cold seeps can also encounter very high HS concentrations. Pore-water HS in cold seeps has been measured at 8 mmol l^{-1} in the Gulf of Mexico (Nix *et al.* 1995) and at 19 mmol l^{-1} in Monterey Bay, USA, with average values in the centers of two Monterey Bay cold seeps of 7.2 and 9.5 mmol l^{-1} (Barry *et al.* 1997). In contrast, even though the HS concentration of the hot hydrothermal vent effluents can exceed 5 mmol l^{-1}, the HS concentration in the 'mixing zone', where most HS-adapted animals are found, averages 300 μmol l^{-1} (for a review, see Fisher, 1995).

The high HS concentrations in cold seeps may give the first indication that there is a limit to HS tolerance. Although vesicomyid clams in Monterey Bay cold seeps typically appeared to utilize nearly 100 % of the available space at sites where HS was detected, the central areas with the highest HS concentrations (7–9 mmol l^{-1} or more) were often barren, with few or no clams, suggesting that these HS concentrations exceed their physiological tolerance (Barry *et al.* 1997). If we assume that the effect of HS on vesicomyid muscle is similar to that on *U. caupo* muscle (K_d between 3 and 30 mmol l^{-1}), then muscle contraction amplitude in vesicomyid clams at the centers of these seeps would be inhibited by at least 25 %. Of course, this assumes that vesicomyid tissue HS concentrations would be similar to those of the pore water, which may not be the case, depending on tissue-based HS sequestration and oxidation. Additionally, muscle tissue of animals adapted to high-HS environments may be found to have a lower sensitivity to HS than that of *U. caupo*. In either case, the possibility that a cytochrome-independent effect of HS on muscle activity limits the distribution of animals in high-HS environments is worthy of further study.

Possible mechanisms of HS action

The mechanism by which HS inhibited muscle contraction in our experiments is unknown. Because the K_d of HS in our experiments was similar whether the muscle was depolarizing spontaneously or being depolarized by field stimulation, the site of action of HS must be in the muscle itself and not in the nerve or at the neuromuscular junction. However, field stimulation of muscle still relies on ion channel conduction, intracellular second messengers, shortening of the actin–myosin complex and, after a sufficient number or duration of contractions, the continued production of ATP and the proper functioning of ion pumps.

Thus, one possible explanation for our results is that high HS

concentrations interfere with glycolysis, with decreased muscle contraction amplitude therefore resulting from depletion of ATP. However, two arguments make this possibility less likely. First, while nerve and muscle in *U. caupo* almost certainly share the same glycolytic enzymes, nerve tissue activity was apparently unaffected by the same HS concentrations that significantly affected muscle contraction. It should be noted, however, that we did not record nerve membrane potential directly, and thus we do not know the K_d for HS on nerve activity. A second argument against inhibition of glycolysis is that, although long-term exposures of body wall muscle to 10 mmol l^{-1} HS decreased the amplitude of contraction to less than half the normal level, this amplitude persisted for at least 6 h. At a frequency of approximately 6 contractions min^{-1}, this period must have constituted over 2000 contractions. In the field stimulation experiments, by comparison, exposure of a muscle strip to an HS concentration well above the K_d completely inhibited the ability of the strip to contract within minutes and before more than one contraction had been evoked (data not shown). Thus, it is unlikely that the inability to contract was due simply to depletion of high-energy substrates.

It is possible that the effect of HS exposure on muscle is at least partially due to decreased intracellular pH. At pH 7.4, HS exists primarily as H_2S and HS^- (the relative concentration of S^{2-} is very low and probably insignificant). Since H_2S is uncharged, this species is assumed to be much more permeable across cell membranes than is either of the charged species (Julian and Arp, 1992). During HS exposure, therefore, the influx of H_2S without accompanying HS^- or S^{2-} is expected to cause intracellular acidification, although this has only been confirmed in large multinucleate algal cells (Jacques, 1936). In vertebrates, an intracellular acidification by 0.5–1 pH unit can lead to either an increase (Burdyga *et al.* 1996) or a decrease (Taggart *et al.* 1994) in contractile force in smooth muscle and only a slight decrease in contractile force in striated muscle (Lamb and Stephenson, 1994). The extent to which HS exposure caused intracellular acidification of *U. caupo* muscle and whether this acidification could have caused inhibition of contraction is not yet known.

Another possibility is suggested by an interesting recent twist in HS research; Abe and Kimura (1996) and Hosoki *et al.* (1997) have proposed that HS may function as an endogenous transmitter in mammalian brain and smooth muscle. In these tissues, HS is produced from L-cysteine by pyridoxal-5′-dependent enzymes, and endogenous HS levels in the mammalian brain have been measured as 50–160 μmol l^{-1}, although the validity of these values has been questioned (Reiffenstein *et al.* 1992). However, the comparatively high K_d of HS toxicity in *U. caupo* muscle makes it extremely unlikely that its actions result from interference with an endogenous HS signaling mechanism. Furthermore, although the K_d for HS action on frequency was somewhat above that on muscle amplitude, it is likely that this apparent toxicity on nerve rhythmicity is caused artificially by the inability of the muscle to respond to a stimulus, since it was only through muscle contraction that nerve frequency was inferred.

We do not have sufficient information to rule out other possible direct sites of HS action. However, the molecular mechanisms proposed for the interactions between HS and hemoglobins may provide a useful starting point. While HS greatly reduces the oxygen affinity of hemoglobins by forming sulfhemoglobin and sulfmyoglobin, in the clam *Solemya reidi*, HS appears to alter cytoplasmic hemoglobin–oxygen reaction kinetics directly, reducing the rate of hemoglobin deoxygenation (Kraus *et al.* 1996). In the hemoglobin of the hydrothermal vent tube worm *Riftia pachyptila*, HS is known to bind reversibly to a site separate from the heme oxygen-binding site. It has been suggested that HS binding in this case occurs at disulfide bridges *via* thiol–disulfide exchange, in which an oxidized disulfide in a protein undergoes nucleophilic attack by a reduced thiol (Arp *et al.* 1987). This reaction could be readily reversible, since a change in HS concentration could allow the protein-disulfide to reform. A similar action at an unknown target in muscle might reversibly inhibit contraction, as is seen in our study. Further experiments will be required to determine the mechanism of HS toxicity in muscle, whether HS-mediated muscle toxicity limits the distribution of animals in high-HS environments and whether HS has additional, as yet undiscovered, toxic effects.

This research was supported by NSF IBN-9218732 and NIH MBRS S06 GM52588-01-7 to A.J.A. We are grateful to Oscar Lang and Jennifer Buelna for help conducting these studies and to members of the RTC-SFSU Ecophysiology Laboratory for help collecting and maintaining animals, and we thank the two anonymous reviewers for helpful comments on this manuscript.

References

ABE, K. AND KIMURA, H. (1996). The possible role of hydrogen sulfide as an endogenous neuromodulator. *J. Neurosci.* **16**, 1066–1071.

ARP, A. J., CHILDRESS, J. J. AND VETTER, R. D. (1987). The sulphide-binding protein in the blood of the vestimentiferan tube-worm, *Riftia pachyptila*, is the extracellular hemoglobin. *J. exp. Biol.* **128**, 139–158.

ARP, A. J., HANSEN, B. M. AND JULIAN, D. (1992). The burrow environment and coelomic fluid characteristics of the echiuran worm *Urechis caupo* from three northern California population sites. *Mar. Biol.* **13**, 613–623.

ARP, A. J., MENON, J. G. AND JULIAN, D. (1995). Multiple mechanisms provide tolerance to environmental sulfide in *Urechis caupo*. *Am. Zool.* **35**, 132–144.

BARRY, J. P., KOCHEVAR, R. E. AND BAXTER, C. H. (1997). The influence of pore-water chemistry and physiology on the distribution of vesicomyid clams at cold seeps in Monterey Bay: Implications for patterns of chemosynthetic community organization. *Limnol. Oceanogr.* **42**, 318–328.

BEAUCHAMP, R. O., BUS, J. S., POPP, J. A., BOREIKO, C. J. AND ADJELKOVICH, D. A. (1984). A critical review of the literature on hydrogen sulfide toxicity. *Crit. Rev. Toxicol.* **13**, 25–97.

BECK, J. F., DONINI, J. C. AND MANECKJEE, A. (1982). The effect of sulfide and cyanide on nerve function. *Toxicol. Lett.* **10**, 189–193.

BECK, J. F., DONINI, J. C. AND MANECKJEE, A. (1983). The influence of sulfide and cyanide on axonal function. *Toxicol.* **26**, 37–45.

BHAMBHANI, Y., BURNHAM, R., SNYDMILLER, G. AND MACLEAN, I. (1997). Effects of 10-ppm hydrogen sulfide inhalation in exercising men and women. Cardiovascular, metabolic and biochemical responses. *J. occup. env. Med*. **39**, 122–129.

BURDYGA, T. V., TAGGART, M. J. AND WRAY, W. (1996). An investigation into the mechanism whereby pH affects tension in guinea-pig ureteric smooth muscle. *J. Physiol., Lond.* **493**, 865–876.

BURN, J. H. AND RAND, M. J. (1960). The relation of circulating noradrenaline to the effect of sympathetic stimulation. *J. Physiol., Lond.* **150**, 295–305.

CHILDRESS, J. J. (1995). Life in sulfidic environments – historical perspective and current research trends. *Am. Zool*. **35**, 83–90.

FENCHEL, T. M. AND RIEDL, R. J. (1970). The sulfide system: a new biotic community underneath the oxidized layer of marine sand bottoms. *Mar. Biol*. **7**, 255–268.

FISHER, C. R. (1995). Toward an appreciation of hydrothermal-vent animals: their environment, ecological physiology, and tissue stable isotope values. In *Sea Floor Hydrothermal Systems: Physical, Chemical, Biological and Geological Interactions* (ed. S. Humphris, R. Zierenberg, L. Mullineaux and R. Thomson), pp. 297–316. Geophysical Monographs Series 91.

FISHER, W. K. AND MACGINITIE, G. E. (1928). A new echiuroid worm from California. *Ann. Mag. nat. Hist*. **10**, 199–204.

GLANTZ, S. A. (1997). *Primer of Biostatistics*. 4th edn. New York: McGraw Hill.

GREER, J. J., REIFFENSTEIN, R. J., ALMEIDA, A. F. AND CARTER, J. E. (1995). Sulfide-induced perturbations of the neuronal mechanisms controlling breathing in rats. *J. appl. Physiol*. **78**, 433–440.

GROENENDAAL, M. (1980). Tolerance of the lugworm (*Arenicola marina*) to sulphide. *Neth. J. Sea Res*. **14**, 200–207.

HANNAH, R. S. AND ROTH, S. H. (1991). Chronic exposure to low concentrations of hydrogen sulfide produces abnormal growth in developing cerebellar Purkinje cell. *Neurosci. Lett*. **122**, 225–228.

HILLE, B. (1992). *Ionic Channels of Excitable Membranes*, 2nd edn. Sunderland, MA: Sinauer Associates.

HOSOKI, R., MATSUKI, N. AND KIMURA, H. (1997). The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. *Biochem. biophys. Res. Commun*. **237**, 527–531.

JACQUES, A. G. (1936). The kinetics of penetration. XII. Hydrogen sulphide. *J. gen. Physiol.* **19**, 397–419.

JULIAN, D. AND ARP, A. J. (1992). Sulfide permeability in the marine invertebrate *Urechis caupo*. *J. comp. Physiol.* B **162**, 59–67.

JULIAN, D., PASSMAN, W. E. AND ARP, A. J. (1996). Water lung and body wall contributions to respiration in an echiuran worm. *Respir. Physiol*. **106**, 187–198.

KOMBIAN, S. B., REIFFENSTEIN, R. J. AND COLMERS, W. F. (1993). The actions of hydrogen sulfide on dorsal raphe serotonergic neurons *in vitro*. *J. Neurophysiol*. **70**, 81–96.

KRAUS, D. W., DOELLER, J. E. AND POWELL, C. S. (1996). Sulfide may directly modify cytoplasmic hemoglobin deoxygenation in *Solemya reidi* gills. *J. exp. Biol.* **199**, 1343–1352.

KRUSZYNA, H., KRUSZYNA, R. AND SMITH, R. P. (1985). Cyanide and sulfide interact with nitrogenous compounds to influence the relaxation of various smooth muscles. *Proc. Soc. exp. Biol. Med*. **179**, 44–49.

LAMB, G. D. AND STEPHENSON, D. G. (1994). Effects of intracellular pH and [Mg^{2+}] on excitation–contraction coupling in skeletal muscle fibres of the rat. *J. Physiol., Lond.* **478**, 331–339.

LAWRY, J. V. (1966). Neuromuscular mechanisms of burrow irrigation in the echiuroid worm *Urechis caupo* (Fisher and MacGinite). II. Neuromuscular activity of dissected preparations. *J. exp. Biol*. **45**, 357–368.

LEE, R. W., KRAUS, D. W. AND DOELLER, J. E. (1996). Sulfide-stimulation of oxygen consumption rate and cytochrome reduction in gills of the estuarine mussel *Geukensia demissa*. *Biol. Bull. mar. Biol. Lab., Woods Hole* **191**, 421–430.

LEVITT, J. M. AND ARP, A. J. (1991). The effects of sulfide on the anaerobic metabolism of two congeneric species of mudflat clams. *Comp. Biochem. Physiol*. **98**B, 339–347.

NATIONAL RESEARCH COUNCIL, SUBCOMMITTEE ON HYDROGEN SULFIDE, DIVISION OF MEDICAL SCIENCES (1979). *Hydrogen Sulfide*. Baltimore, MD: University Park Press.

NICHOLLS, P. (1975). The effect of sulfide on cytochrome aa3 isosteric and allosteric shifts of the reduced a-peak. *Biochim. biophys. Acta* **396**, 24–35.

NIX, E. R., FISHER, C. R., VODENICHAR, J. AND SCOTT, K. M. (1995). Physiological ecology of a mussel with methanotrophic endosymbionts at three hydrocarbon seep sites in the Gulf of Mexico. *Mar. Biol.* **122**, 605–617.

OESCHGER, R. AND STOREY, K. B. (1990). Regulation of glycolytic enzymes in the marine invertebrate *Halicryptus spinulosis* (Priapulida) during environmental anoxia and exposure to hydrogen sulfide. *Mar. Biol*. **106**, 261–266.

PILGER, J. F. (1993). Echiura. In *Microscopic Anatomy of Invertebrates*, vol. 12, *Onychophora, Chilopodia and Lesser Protostomata* (ed. F. W. Harrison and E. E. Ruppert), pp. 185–236. New York: Wiley-Liss.

POWELL, M. A. AND SOMERO, G. N. (1986). Adaptations to sulfide by hydrothermal vent animals: sites and mechanisms of detoxification and metabolism. *Biol. Bull. mar. Biol. Lab., Woods Hole* **171**, 274–290.

REIFFENSTEIN, R. J., HULBERT, W. C. AND ROTH, S. H. (1992). Toxicology of hydrogen sulfide. *A. Rev. Pharmac. Toxicol*. **32**, 109–134.

ROTH, S. H., SKRAJNY, B. AND REIFFENSTEIN, R. J. (1995). Alteration of the morphology and neurochemistry of the developing mammalian nervous system by hydrogen sulfide. *Clin. exp. Pharmac. Physiol.* **22**, 379–380.

SOMERO, G. N., CHILDRESS, J. J. AND ANDERSON, A. E. (1989). Transport, metabolism and detoxification of hydrogen sulfide in animals from sulfide-rich marine environments. *Crit. Rev. aquat. Sci.* **1**, 591–614.

TAGGART, M., AUSTIN, C. AND WRAY, S. (1994). A comparison of the effects of intracellular and extracellular pH on contraction in isolated rat portal vein. *J. Physiol., Lond.* **475**, 285–292.

THEEDE, H., PONAT, A., HIROKI, K. AND SCHLIEPER, C. (1969). Studies on the resistance of marine bottom invertebrates to oxygen-deficiency and hydrogen sulphide. *Mar. Biol*. **2**, 325–337.

VETTER, R. D., WELLS, M. E., KURTZMAN, A. L. AND SOMERO, G. N. (1987). Sulfide detoxification by the hydrothermal vent crab *Bythograea thermydron* and other decapod crustaceans. *Physiol. Zool*. **60**, 121–137.

VISMANN, B. (1991). Sulfide tolerance: Physiological mechanisms and ecological implications. *Ophelia* **34**, 1–27.

VÖLKEL, S. AND GRIESHABER, M. K. (1996). Mitochondrial sulfide oxidation in *Arenicola marina*. Evidence for alternative electron pathways. *Eur. J. Biochem*. **235**, 231–237.

ZAR, J. H. (1984). *Biostatistical Analysis*. Englewood Cliffs, NJ: Prentice-Hall.